PI: **KREIPKE, CHRISTIAN W**	Title: Molecular Mechanisms of Enhanced Contractility following Traumatic Brain Injury:	
Received: 03/13/2009	FOA: PA07-070	Council: 10/2009
Competition ID: ADOBE-FORMS-A	FOA Title: RESEARCH PROJECT GRANT (PARENT R01)	
1 R01 NS064976-01A2	Dual:	Accession Number: 3149442
IPF: 9110501	Organization: WAYNE STATE UNIVERSITY	
Former Number:	Department: Anatomy and Cell Biology	
IRG/SRG: ANIE	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 250,000 Year 2: 250,000 Year 3: 250,000 Year 4: 250,000 Year 5: 250,000	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Y Early Stage Investigator: Y

Senior/Key Personnel:	*Organization:*	*Role Category:*
Christian Kreipke Ph.D.	Wayne State University	PD/PI
Jose Rafols PhD	Wayne State University	Faculty
Donald Kuhn PhD	Wayne State Unversity	Faculty
Patrick Mueller PhD	Wayne State University	Faculty
Ewart Haacke PhD	Wayne State University	Consultant

Appendices

Jcmm cp review

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	**State Application Identifier**

1. * TYPE OF SUBMISSION

☐ Pre-application ☐ Application ☒ Changed/Corrected Application

4. Federal Identifier NS064976

5. APPLICANT INFORMATION

*** Organizational DUNS:** 001962224

* Legal Name: Wayne State University

Department: | Division:

* Street1: 5057 Woodward

Street2:

* City: Detroit | County:

* State: MI: Michigan | Province:

* Country: USA: UNITED STATES | * ZIP / Postal Code: 48202

Person to be contacted on matters involving this application

Prefix: Ms. | * First Name: Carole | Middle Name:

* Last Name: Bach | Suffix:

* Phone Number: 313.577.2294 | Fax Number: 313.577.2653

Email: orpsmail@wayne.edu

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN)*: 38-6028429

7. * TYPE OF APPLICANT: H: Public/State Controlled Institution of Higher Education

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☐ New ☒ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER:

TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Molecular Mechanisms of Enhanced Contractility following Traumatic Brain Injury: Towards a Clinical Trial

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*: Detroit, Wayne, Michigan

13. PROPOSED PROJECT:

* Start Date	* Ending Date
11/01/2009	10/31/2014

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant	b. * Project
MI-013	MI-013

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. | * First Name: Christian | Middle Name: William

* Last Name: Kreipke | Suffix: Ph.D.

Position/Title: Assistant Professor

* Organization Name: Wayne State University

Department: Anatomy and Cell Biology | Division: Medicine

* Street1: 540 E. Canfield

Street2:

* City: Detroit | County: Wayne

* State: MI: Michigan | Province:

* Country: USA: UNITED STATES | * ZIP / Postal Code: 48201

* Phone Number: 313.577.1049 | Fax Number: 313.577.3125

* Email: ckreipke@med.wayne.edu

OMB Number: 4040-0001
Expiration Date: 04/30/2008

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

16. ESTIMATED PROJECT FUNDING

a. * Total Estimated Project Funding	1,900,000.00
b. * Total Federal & Non-Federal Funds	1,900,000.00
c. * Estimated Program Income	0.00

17. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

18. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

19. Authorized Representative

Prefix:	Ms.
* First Name:	April
Middle Name:	
* Last Name:	Spraggins
Suffix:	
* Position/Title:	Pre-Award Officer
* Organization:	Wayne State University
Department:	Sponsored Programs Administrat
Division:	Pre-Award
* Street1:	4201 St. Antoine
Street2:	
* City:	Detroit
County:	USA
* State:	MI: Michigan
Province:	
* Country:	USA: UNITED STATES
* ZIP / Postal Code:	48201
* Phone Number:	313.577.9848
Fax Number:	313.577.1348
* Email:	aspraggi@med.wayne.edu

*** Signature of Authorized Representative**: April Spraggins

*** Date Signed**: 03/13/2009

20. Pre-application Add Attachment | Delete Attachment | View Attachment

21. Attach an additional list of Project Congressional Districts if needed.

Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

424 R&R and PHS-398 Specific Table Of Contents

Appendix

Number of Attachments in Appendix: 1

RESEARCH & RELATED Project/Performance Site Location(s)

Project/Performance Site Primary Location

Organization Name: Wayne State University
* Street1: 540 E Canfield
Street2: Room 9312, 9320, 9332
* City: Detroit
County: Wayne
* State: MI: Michigan
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 48201

Project/Performance Site Location 1

Organization Name:
* Street1:
Street2:
* City:
County:
* State:
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code:

Additional Location(s) Add Attachment | Delete Attachment | View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian, William

Close Form | Print Page | About

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Exemption Number: ☐1 ☐2 ☐3 ☐4 ☐5 ☐6

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☐ Yes ☒ No

IACUC Approval Date: 02/01/2008

Animal Welfare Assurance Number A3310-01

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5.a. * Does this project involve activities outside the U.S. or partnership with International Collaborators? ☐ Yes ☒ No

5.b. If yes, identify countries:

5.c. Optional Explanation:

6. * Project Summary/Abstract 1234-Abstract-A2.pdf Add Attachment | Delete Attachment | View Attachment

7. * Project Narrative 1238-Project Narrative.pdf Add Attachment | Delete Attachment | View Attachment

8. Bibliography & References Cited 1237-Cp References-A2.pdf Add Attachment | Delete Attachment | View Attachment

9. Facilities & Other Resources 1235-Resources.pdf Add Attachment | Delete Attachment | View Attachment

10. Equipment 1236-Equipment.pdf Add Attachment | Delete Attachment | View Attachment

11. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

OMB Number: 4040-0001
Expiration Date: 04/30/2008

Abstract

Traumatic brain injury (TBI) is reportedly the leading cause of death and disability among children and young adults (CDC Report, 2004). Among multiple sequelae, TBI results in three major pathologies: 1) cerebral edema which leads to a critical rise in intracranial pressure, 2) diffuse axonal injury which brings about disruption of neural circuits underlying cognitive and motoric behaviors, and 3) alterations in the brain's microcirculation that cause a persistent state of hypoperfusion and improper delivery of vital metabolites to neural tissue. Over 25 clinical trials aimed at the first two pathologies have been developed, none of which have been effective in the treatment for TBI. Therefore, novel studies leading to new clinical trials are necessary. To date no one has initiated a clinical trial addressing the third pathology, *dysfunctional vascular reactivity following TBI*. The present proposal provides rationale for proceeding towards a clinical trial by implementing novel strategies that aim to improve cerebral blood flow (CBF) and cognitive outcome following TBI. While our laboratory has published extensively on the role of endothelin-1 in mediating altered cerebral vascular reactivity after TBI, the cellular and molecular **mechanism** for this altered vasoreactivity remains to be elucidated. In addition **the causal relationship between ET-1, altered vasoreactivity and functional outcome has not been established**. This proposal addresses these issues by pharmacologic manipulation of the ET-1 system and calponin (Cp), a key element in vasoreactivity—the molecular events leading to vascular smooth muscle contractility and hence to vasoconstriction. **The central hypothesis of this proposal is: *TBI causes enhanced endothelin-1-mediated vasoconstriction and reduced CBF, which, in turn, exacerbates TBI-induced neuronal injury and cognitive deficits***.

Project Narrative

Traumatic brain injury (TBI) is the leading cause of death and disability amongst our youth and children. Further, it has been named as the signature injury in the War on Terrorism that, upon return of our men and women fighting in Iraq and Afghanistan, is projected to cost millions in patient care and rehabilitation costs. While TBI results in three major pathologies, including diffuse axonal injury, brain edema, and hypoperfusion of the brain's parenchyma, this proposal investigates novel methods to increase blood flow after injury by investigating the fundamental mechanism behind hypoperfusion. In doing so, the experiments in this proposal are designed to yield results that can quickly be translated into the clinical setting, thus off-setting the current potentially dismal outcome following exposure to TBI.

Laboratory:

The laboratory consists of approximately 700 sq.ft. in which animal perfusion, tissue processing, sectioning and analysis can be performed. There is also an equipment room where the surgeries are conducted and which houses the trauma model. Dr. Kreipke has additional laboratory of 700 sq.ft. that is used for behavioral testing.

Animal:

Facilities for the care and housing of experimental animals are available in the basement of Scott Hall. These resources are operated by the University Department of Laboratory Animal Resources. At WSU, all animals used for biomedical research at the medical center are housed in modern animal care facilities with excellent supervisory and veterinary support.

Computer:

Three Dell Dimension 8400 and One Toshiba Portege; 1 Epson Photo r100 printer.

Office:

The office facilities are located adjacent to the laboratory. The Principal Investigator and Co-investigator have their own office space fully equipped with computers and computer peripheria.

Equipment:

Three cryostats are available in the Department of Anatomy and Cell Biology; balance, ph meter, freezer, 2 refrigerators, dessicator, and equipment for ICC and Western blots are also available. Further equipment related to molecular biology is avialable to us from Dr. Kuhn's laboratory. All behavioral equipment including raidal arm maze, Morris water maze, treadmills, motor testing equipment are also currently available. Dr. Haacke, as indicated in his letter of support, will provide full access to MRI facilities.

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. * First Name: Christian Middle Name: William
* Last Name: Kreipke Suffix: Ph.D.
Position/Title: Assistant Professor Department: Anatomy and Cell Biology
Organization Name: Wayne State University Division: Medicine
* Street1: 540 E. Canfield
Street2:
* City: Detroit County: Wayne
* State: MI: Michigan Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 48201
* Phone Number: 313.577.1049 Fax Number: 313.577.3125
* E-Mail: ckreipke@med.wayne.edu

Credential, e.g., agency login: aa5930

*** Project Role:** PD/PI **Other Project Role Category:**

***Attach Biographical Sketch** 1246-X-biosketch-Kreipke.pdf Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support 1247-Ongoing Research Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 1

Prefix: Dr. * First Name: Jose Middle Name:
* Last Name: Rafols Suffix: PhD
Position/Title: Professor Department: Anatomy and Cell Biology
Organization Name: Wayne State University Division: Medicine
* Street1: 540 E. Canfield
Street2:
* City: Detroit County: USA
* State: MI: Michigan Province:
* Country: USA: UNITED STATES * Zip / Postal Code: 48201
* Phone Number: 313.577.0574 Fax Number: 313.577.3125
* E-Mail: jrafols@med.wayne.edu

Credential, e.g., agency login: aa3302

*** Project Role:** Faculty **Other Project Role Category:**

***Attach Biographical Sketch** 1248-X-biosketch-Rafols.pdf Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support 1249-Ongoing Research Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 2

Prefix: Dr. * First Name: Donald Middle Name:

* Last Name: Kuhn Suffix: PhD

Position/Title: Professor Department: Psychiatry

Organization Name: Wayne State Unversity Division: Medicine

* Street1: 4646 John R

Street2:

* City: Detroit County: Wayne

* State: MI: Michigan Province:

* Country: USA: UNITED STATES * Zip / Postal Code: 48201

* Phone Number: 313.576.4457 Fax Number:

* E-Mail: donald.kuhn@wayne.edu

Credential, e.g., agency login: aa3071

*** Project Role:** Faculty **Other Project Role Category:**

***Attach Biographical Sketch** 1250-X-biosketch-Kuhn.pdf Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support 1251-Ongoing Research Support Add Attachment Delete Attachment View Attachment

PROFILE - Senior/Key Person 3

Prefix: Dr. * First Name: Patrick Middle Name:

* Last Name: Mueller Suffix: PhD

Position/Title: Assistant Professor Department: Physiology

Organization Name: Wayne State University Division: Medicine

* Street1: 540 E Canfield

Street2:

* City: Detroit County: Wayne

* State: MI: Michigan Province:

* Country: USA: UNITED STATES * Zip / Postal Code: 48201

* Phone Number: 313-577-1559 Fax Number:

* E-Mail: pmueller@med.wayne.edu

Credential, e.g., agency login: dq4607

*** Project Role:** Faculty **Other Project Role Category:**

***Attach Biographical Sketch** 1252-X-biosketch-Mueller.pdf Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Senior/Key Person 4

Prefix: Dr. * First Name: Ewart Middle Name: Mark

* Last Name: Haacke Suffix: PhD

Position/Title: Professor Department: Radiology

Organization Name: Wayne State University Division: Medicine

* Street1: 3990 John R

Street2: MRI concourse

* City: Detroit County: Wayne

* State: MI: Michigan Province:

* Country: USA: UNITED STATES * Zip / Postal Code: 48201

* Phone Number: 313.745.1395 Fax Number:

* E-Mail: NMRIMAGING@aol.com

Credential, e.g., agency login: ak5444

*** Project Role:** Consultant **Other Project Role Category:**

***Attach Biographical Sketch** 1253-X-biosketch-Haacke.pdf Add Attachment Delete Attachment View Attachment

Attach Current & Pending Support 1254-Ongoing Research Support Add Attachment Delete Attachment View Attachment

ADDITIONAL SENIOR/KEY PERSON PROFILE(S) Add Attachment Delete Attachment View Attachment

Additional Biographical Sketch(es) (Senior/Key Person) Add Attachment Delete Attachment View Attachment

Additional Current and Pending Support(s) Add Attachment Delete Attachment View Attachment

OMB Number: 4040-0001
Expiration Date: 04/30/2008

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Christian W. Kreipke	POSITION TITLE Assistant Professor, Research
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Wayne State University	B.A.	1995-1999	Anthropology
Wayne State University	M.A.	1999-2000	Medical Anthropology
Wayne State University, School of Medicine	Ph.D.	2000-2004	Neuroscience

A. Positions and Honors

01/97-05/97 Wayne State University, School of Medicine and Hutzel Hospital, Research Assistant, Bone Densitometry/Osteoporosis Project

09/97-09/99 Wayne State University, Institute for Information and Technology, Research Assistant, HIV/AIDS in Detroit Project

09/99-05/00 Wayne State University, Graduate Teaching Assistant, Department of Anthropology

05/00-09/00 Wayne State University, Adjunct Instructor, Department of Anthropology

09/00-08/04 Wayne State University, School of Medicine, Pre-Doctoral Research Assistant, National Institute of Drug Abuse T32 Training Grant

08/04-04/08 Wayne State University, School of Medicine, Research Associate, Dept. Anatomy and Cell Biology, Traumatic Brain Injury

04/08-present Wayne State University, School of Medicine, Research Scientist, Dept. Anatomy and Cell Biology

Other Experience and Professional Memberships

05/99-present Member, Phi Beta Kappa

02/00-present Member, Society for Applied Anthropology

02/00-present Member, Society for Medical Anthropology

05/01-present Member, Sigma Xi

05/01-present Member, New York Academy of Sciences

03/01-03/02 Society for Neuroscience Brain Awareness Week Committee, Wayne State University, Chair

05/02-present Member, Society for Neuroscience

05/02-05/04 Michigan Society for Neuroscience, Student Counselor

05/03 Michigan Society for Neuroscience Chapter Meeting coordinator

11/04-08/07 Sigma Xi, Wayne State Chapter, Executive Board Member

02/05-08/07 Wayne State Alumni Communications Committee, Committee Member

05/06-08/07 Sigma Xi, National, Associate Director, NorthCentral Region

03/07-present Member, International Society for Cerebral Blood Flow and Metabolism

02/07-present Chairman of the Board, Southfield Oncology Institute

08/07-present Sigma Xi, National, Acting Director, NorthCentral Region

02/08-present Full Member of The Royal Society

Honors

2002 Dean Thomas Asselin, M.D. Endowed Prize for Excellence in Psychiatry and Behavioral Neuroscience Research (Wayne State University School of Medicine)

2003 1st Place, Society for Neuroscience, MI Chapter, Poster Award

2006 Service Award For 2006 Sigma Xi National Conference

2007 Travel Award, Brain '07, Society for Cerebral Blood Flow and Metabolism

2007 Young Investigators Award, Endothelin 10, Endothelin

B. Peer-reviewed publications (in chronological order)

1. Kuhn DM, Sadidi M, Lu X, Kreipke C, Geddes T, Borges C, and Throck J. 2002 Peroxynitrite-Induced Nitration of Tyrosine Hydroxylase: Identification of Tyrosines 423, 428, and 432 as Sites of Modification by MALDI-TOF Mass Spectrometry and Tyrosine-Scanning Mutagenesis. Journal of Biological Chemistry 277:14336-14342.

2. Kreipke C, Walker PD. 2004. NMDA receptor blockade attenuates locomotion elicited by intrastriatal dopamine D1-receptor stimulation. Synapse 53:25-32.

3. Kreipke C, Rosenberg D, Keshavan M. 2004. Does disordered brain development cut across diagnostic boundaries? In Keshavan M, Kennedy J, Murray R (Eds.) Neurodevelopment and Schizophrenia. Cambridge University Press.

4. Kreipke C, Rafols J, Petrov T. 2005. Transcriptional and translational mechanisms for the reciprocal control of iNOS and endothelin 1 expression in brain microvessels after traumatic brain injury (TBI). Journal of Cerebral Blood Flow and Metabolism 25, S191.

5. Kreipke CW, Campbell BM, Walker PD. 2005. Failure of MK-801 to suppress D1 receptor-mediated induction of locomotor activity and striatal preprotachykinin mRNA expression in the dopamine-depleted rat. Neuroscience 137:505-517.

6. Kreipke CW, Morgan N, Petrov T, Rafols J. 2006. Calponin and caldesmon cellular domains in reacting microvessels following traumatic brain injury. Microvas Res. 71:197-204.

7. Shen Y, Kou Z, Kreipke CW, Petrov T, Hu J, Haacke EM. 2006. In vivo measurement of tissue damage, oxygen saturation changes and blood flow changes after experimental traumatic brain injury in rats using susceptibility-weighted imaging. Magn Reson Imaging 25(2):219-227.

8. Kreipke CW, Morgan R, Petrov T, Rafols JA. 2007. Subcellular Redistribution of Calponin Underlies Sustained Vascular Contractility Following Traumatic Brain Injury. Neurol Res. 29:604-609.

9. Petrov T, Kreipke C, Alilain W, Nantwi K. 2007. Differential Expression Adenosine A1 and A2 Receptor Protein Levels Following Upper Cervical (C2) Spinal Cord Hemisection In Adult Rats. J Spinal Cord Med 30:331-337.

10. Rafols J., Kreipke C, Petrov T. 2007. Alterations in Cerebral Cortex Microvessels and the Microcirculation in a Rat Model of Traumatic Brain Injury: a Correlative EM and Laser Doppler Flowmetry Study. Neurol Res 29:339-347.

11. Rafols J, Morgan R, Kallikuri S, Kreipke C. 2007. Extent of nerve cell injury in Marmarou's model compared to other brain trauma models. Neurol Res 29:348-355.

12. Degracia D, Kreipke C, Kayali F, Rafols JA. 2007. Brain endothelial HSP-70 stress response coincides with endothelial and pericyte death after brain trauma. Neurol Res 29:356-361.

13. Kallukuri S, Kreipke C, Rossi NF., Rafols JA, Petrov T. 2007. Spatial alterations in endothelin receptor expression are temporally associated with the altered microcirculation after brain trauma Endothelin receptor localization following traumatic brain injury. Neurol Res 29:362-368.

14. Kreipke C, Morgan R, Roberts G, Bagchi M, Rafols JA. 2007. Calponin phosphorylation in cerebral cortex microvessels mediates sustained vasoconstriction after brain trauma. Neurol Res 29:369-374.

15. Morgan R, Kreipke C, Robert G, Bagchi M, Rafols J. 2007. Neovascularization following traumatic brain injury: possible evidence for both angiogenesis and vasculogenesis. Neurol Res 29:375-381.

16. Kreipke CW, Morgan R, Kallakuri S, Rafols JA. 2007. Behavioral pre-conditioning enhances angiogenesis and cognitive outcome after brain trauma. Neurol Res. 29:388-94.

17. Dore-Duffy P, Kreipke C, Rafols JA. 2007. Differential expression of capillary VEGF isoforms following traumatic brain injury. Neurol Res 29:395-403.

18. Petrov T, Kreipke C, Alilain W, Nantwi KD. 2007. Differential expression of adenosine A1 and A2A receptors after upper cervical (C2) spinal cord hemisection in adult rats. J Spinal Cord Med. 30:331-337.

19. Huttemann M, Lee I, Kreipke CW, Petrov T. (in press). Suppression of iNOS prior to traumatic brain injury improves cytochrome oxidase activity and normalizes cellular energy levels. Neuroscience

20. Kreipke CW, Schafer PC, Rafols JA. 2008. Endothelin receptor A antagonism ameliorates hypoperfusion and enhances cognitive outcome following traumatic brain injury. Brain Injury 22:S43.

21. Rafols JA, Kreipke CW, Kallakuri S. 2008. Upregulation of endothelin-1 receptors in neurons and brain microvessels coincides temporally with a dysfunctional microcirculation after traumatic brain injury. Brain Injury 22:S44.

22. Kreipke CW, Schafer PC, Michael D, Rafols JA. (in press). Endothelin receptors A and B are expressed in distinct cellular compartments of rat hippocampus following global ischemia: an immunocytochemical study. Can J Physio Pharm.

23. Hoffman WH, Stamatovic SM, Rafols JA, Kreipke CW, Andjelkovic AV. (in press). Inflammatory mediators and blood brain barrier disruption in fatal brain edema of diabetic ketoacidosis. Experimental Diabetes Research.

24. Kreipke CW, Schafer PC, Rossi NF, Rafols JA. (in press). Differential affects of Endothelin receptor-A and B antagonism on hypoperfusion following traumatic brain injury (TBI). Neurological Research.

25. Hoffman W, Artlett C, Zhang W, Kreipke CW, Passmore G, Rafols JA, Sima AA. (in press) Receptor for advanced glycation end products and neuronal deficit in the fatal brain edema of diabetic ketoacidosis. Brain Research.

26. Kreipke CW, Rafols JA. (in press). Calponin control of cerebrovascular reactivity: Therapeutic implications in brain trauma. J Cell Mol Med.

27. Schafer PC, Schafer SM, Kreipke CW. (in press). Effects of light-dark cycle on motoric and cognitive activity: Implications for behavioral testing. Bio Behav Res.

C. Research Support

Ongoing Research Support

R01 NS39860 T. Rafols (PI) 3/10/04-4/30/09

NIH-NINDS
Role: CO-I (70% effort)
"Control of microvascular tone in traumatic brain injury"
Investigates the role of endothelin receptors in the control of the microcirculation in a rat model of traumatic brain injury. There is NO overlap between this project and the current proposal. Effort on this project will be reduced to 40% upon successful funding of the current grant.

VA RR & D Award. Rossi (PI) 1/01/08-12/31/11
VA Rehabilitation
Role: CO-I (30% Effort)
"Conditioning, microvascular tone & rehabilitation post brain trauma"
Investigates the role of exercise in the control of microcirculation in a rat model of traumatic brain injury. There is NO overlap between this project and the current proposal.

Principal Investigator/Program Director (Last, First, Middle): Rafols, Jose A.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Jose A. Rafols	Professor
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Illinois Benedictine, Lisle, IL	B.S.	1965	Biology
University of Kansas, Kansas City, KS	Ph.D.	1969	Anatomy
S. Ramon Y Cajal Institute, CSIC, Madrid, Spain	Post Doc	1970	Neuroanatomy

A. Positions and Honors

Positions and Employment

1969-1970	Instructor, Dept. of Anatomy/Cell Biology, Wayne State University, School of Medicine
1970	NIH Postdoctoral trainee at S. Ramon Y Cajal Institute, CSIC, Madrid, Spain
1971-1973	Asst. Professor, Dept. of Anatomy/Cell Biology, Wayne State University, School of Medicine
1973-1989	Assoc. Professor, Dept. of Anatomy/Cell Biology, Wayne State University, School of Medicine
1989-present	Professor, Dept. of Anatomy/Cell Biology, Wayne State University, School of Medicine
1994-present	Dir., Morphology and Imaging Core, Neurotrauma Center, Wayne State University, School of Medicine

Honors

DHHS/PHS/NIH Study Section Member (full member), Neurological Disorder Program Project Review A Committee (NSP-term) 7/1/90-6/30/94.
Chairman, Site visit, The Johns Hopkins University, Baltimore, MD; "Disorders of aging neuro-transmitter systems and neurotrophins", December 15-17, 1991.
Member, National Institutes of Health Reviewers Reserve (NRR), for term 7/1/94-6/30/98.
Member, American Heart Association National Study Committee, Brain Review Committee, for term 7/96-6/99.

B. Selected peer-reviewed publications (past five years)

Petrov T, Page AB, Owen C, Rafols JA. 2000 Expression of the inducible nitric oxide synthase in distince cellular types after traumatic brain injury. Acta Neuropathol 100:196-204.
Dore-Duffy P, Owen C, Bahabanov R, Murphy S, Rafols JA. 2000 Pericyte response to traumatic brain injury (TBI): Elongation and migration from the microvascular wall. Microvascular Res 60:55-69.
White BC, Sullivan JM, DeGracia DJ, O'Neill BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS. 2000 Brain ischemia and reperfusion: Molecular mechanisms of neuronal injury. J Neurolog Sci 179:1-33.
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Goshgarian HG, Rafols JA. 1984 The ultrastructural and synaptic architecture of phrenic motor neurons in the spinal cord of the adult rat. J Neurocytol 13:85-109.

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Lenzi T, Raols JA. 1996 Reperfusion-induced changes in a nine-vessel occlusion model of ischemia. Soc NEurosci (Abstracts) 22:2157.
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Folkerts MM, Berman Wang G, Murphy S, Rafols JA, Muizelaar JP. 1996 Behavior morphological and electrophysiological effects of diffuse axonal injury in rats. J Neurotrauma 13:610.
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Sullivan JM, Alousi SS, Kikade KR, Rafols JA, Krause GS, White BC. 1998 Insulin induces dephosphorylation of eIF2alpha(P) and restores protein synthesis in vulnerable hippocampal neurons following transient ischemia. J Cereb Blood Flow Metab 19:1010-1019.
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Goldstein EN, Own CR, White BC, Rafols JA. 1999 Ultrastructural localization of phosphorylated eIF2(alpha)P during brain reperfusion. Acta Neuropathol 98:493-505.
McKeown TR, Goldstein EN, Sullivan JM, Rafols JA, White BC, Krause GS. 1999 Nuclear localization and DNA binding properties of phosphorylated eIF2alpha (P) ub vulnerable hippocampal neurons during reperfusion. J Cereb Flow Metab 19:S510.
Rafols JA, Alousi SS, Owen CR, White BC, Sullivan JM. 1999 High doses of insulin do not prevent dephosphorylation of eIF2alpha(P), recovery of protein synthesis, and atrophy of hippocampal cA3 neurons during reperfusion. J Cereb Flow Metab 19:s511.
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Underwood BD, Lipinski CA, Rafols JA, Crossland WC, McAllister JP, Diaz FG, White BC. 1999 Effects of traumatic brain injury on phosphorylated eIF2alpha in the rat. Soc. Neurosci (abrstract) 25:820.

C. Ongoing Research Support

VA RR & D Award. Rossi (PI) 1/01/08-12/31/11
VA Rehabilitation
Role: CO-I (20% Effort)
"Conditioning, microvascular tone & rehabilitation post brain trauma"
Investigates the role of exercise in the control of microcirculation in a rat model of traumatic brain injury.

NIH-NINDS RO1 NS39860 06/01/04-05/31/09
Control of Microvascular tone in Traumatic Brain Injury.
The long term objective of this project is to investigate the effects of traumatic brain injury on the gene regulation and synthesis of molecules which effect contractility or relaxation of the smooth muscle cells in the wall of cerebral microvessels. The hypothesis being tested is that altered regulation of the genes that encode for endothelin receptors in endothelial cells, at different time points participate in the abnormal contractility of brain microvessels following trauma.
PI

NIH-NINDS RO1 NS044100 07/01/03-06/30/08
The Unfolded Protein Response after Brain Ischemia.
The proposal aims at investigating the unfolded-protein response as a mechanism of cell death in hippocampal CA1 neurons after brain ischemia/reperfusion.
Co-I

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Donald M. Kuhn	Professor
eRA COMMONS USER NAME	
aa3071	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Presbyterian College	BS	1972	Biopsychology
University of South Carolina	PhD	1976	Behavioral Pharmacology
Princeton University	Postdoc	1976-1977	Electrophysiology
National Institutes of Health	Postdoc	1977-1983	Biochemical Pharmacology

A. Positions and Honors

Positions and Employment

1983-1986-Chief, Section on Biochemical Pharmacology, National Heart Lung & Blood Institute, NIH
1985-1986-Alexander von Humboldt Fellow, Department of Neurochemistry, Goethe University, Frankfurt, Germany
1987-present- Professor, Department of Psychiatry and Behavioral Neurosciences, Center for Molecular Medicine and Genetics, and Institute for Chemical Toxicology, Wayne State University School of Medicine
1993-1994-Visiting Professor, Dept. Molecular Genetics and HHMI, Univ. Texas Southwestern Medical Center, Dallas, Texas (Sabbatical leave in Dr. T. Sudhof's lab)
1998-present-Investigator, John D. Dingell VA Medical Center, Detroit, MI

Other Experience and Professional Memberships

1994-1998 Member, NIDA-C (now NMB) Scientific Review Subcommittee
1998-2002 Member, MDCN-4 Scientific Review Subcommittee
1999- Member, Editorial Board Journal of Neurochemistry
1999- Ad hoc reviewer for MDCN-3, IFCN-7, Neurological Sciences & Disorders B, NIDA Cebra Program, and numerous SEPs for NIDA, NINDS, and NIMH
2001- National Scientific Advisory Council, American Federation for Aging Research
2004- Member, Neurobiology A Merit Review Subcommittee, Dept. Veterans Affairs
2006- Member, NMB Scientific Review Subcommittee

Honors

1985- Fellow, Alexander von Humboldt Foundation

B. Selected peer-reviewed publications (in chronological order)

(Publications selected more than 135 peer-reviewed publications and book chapters)

Kuhn, D.M., Arthur, R., Jr., and Yoon, H., and Sankaran, K. Tyrosine hydroxylase in secretory granules from bovine adrenal medulla: Evidence for an integral membrane bound form. J. Biol. Chem. 265, 5780-5786, 1990.

Wolf, W.A., Zaija. E., Arthur, R.A. Jr., Anastasiadis, P.Z., Levine, R.A., and **Kuhn, D.M.** Effect of tetrahydrobiopterin on serotonin synthesis, release, and metabolism in superfused hippocampal slices. J. Neurochem. 57, 1191-1197, 1991.

Johansen, P.A., Jennings, I., Cotton, R.G.H., and **Kuhn, D.M.** Immobilization of tryptophan hydroxylase by

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Kuhn, D.M., Arthur, R.A., and States, J.C. Phosphorylation and activation of brain tryptophan hydroxylase: Identification of serine-58 as a substrate site for protein kinase A. J. Neurochem. 68, 2220-2223, 1997.
Kuhn, D.M. and Arthur, R.A. Molecular mechanism of the inactivation of tryptophan hydroxylase by nitric oxide: Attack on critical sulfhydryls which spare the enzyme iron center. J. Neurosci.17, 7245-7251, 1997.
Kuhn, D.M. and Arthur, R.E. Dopamine inactivates tryptophan hydroxylase and forms a redox-cycling quinoprotein: Possible endogenous toxin to serotonin neurons. J. Neurosci. 18, 7111-7117, 1998.
Anastasiadis, P.Z., Imerman, B., Louie, M.C., States, J.C., **Kuhn, D.M.**, and Levine, R.A. Tetrahydrobiopterin as obligatory mediator of PC12 cell proliferation induced by EGF and NGF. Eur. J. Neurosci., 9, 1831-1837, 1997.
Kuhn, D.M., Arthur, R.E., Thomas, D.M., and Elferink, L.A. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: Relevance to Parkinson's Disease. J. Neurochem. 73, 1309-1317, 1999.
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Kuhn, D.M., Aretha, C.W. and Geddes, T.J. Peroxynitrite inactivation of tyrosine hydroxylase: Mediation by sulfhydryl oxidation, not tyrosine nitration. J. Neuroscience, 19, 10289-10294, 1999.
Kuhn, D.M. and Geddes, T.J. Molecular footprints of neurotoxic amphetamine action. Annals NY Academy of Sciences, 914, 92-103, 2000.
Anastasiadis, P.Z., Jiang, H., Bezin, L., **Kuhn, D.M.**, and Levine, R.A. Tetrahydrobiopterin enhances apoptotic cell death following withdrawal of trophic support. J. Biol. Chem. 276, 9050-9058, 2001.
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Park, S., Ferrer, J., Javitch, J.A., and **Kuhn, D.M.** Peroxynitrite inactivates the human dopamine transporter by modification of cysteine 342: Potential mechanism of neurotoxicity in dopamine neurons. J. Neuroscience, 22, 4399-4405, 2002.
Kuhn, D.M. Regulation of Tyrosine Hydroxylase by S-glutatiolation: Relevance to Conditions Associated with Dopamine Neuronal Damage, S. Milstien and G. Kapatos (Eds.). In: Pteridines and Folates in Biology and Medicine, Plenum Press, 61-64, 2002.
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tyrosine hydroxylase by peroxynitrite and nitrogen dioxide: Is nitrotyrosine formation an early step in dopamine neuronal damage? J. Biol. Chem, 278, 28736-28742, 2003.

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Thomas, D.M., Verbeem, D., Liu, X., and **Kuhn, D.M.** Identification of differentially regulated transcripts in mouse striatum following methamphetamine treatment: An oligonucleotide microarray approach. J. Neurochemistry, 88, 380-393, 2004.

Albertson, D.N., Pruetz, B., Schmidt, C.J., **Kuhn, D.M.**, Kapatos, G., and Bannon, M.J. Decreased Expression of Myelin-Related Genes in Human Cocaine Abusers. J. Neurochemistry, 88, 1211-1219, 2004.

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Thomas, D.M., Dowgiert, J., Geddes, T.J., Verbeem, D., Liu, X., and **Kuhn, D.M.** Microglial activation is a pharmacologically specific marker for the neurotoxic amphetamines. Neurosci. Lett., 367, 349-354, 2004.

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Thomas, D.M. and **Kuhn, D.M.** Attenuated microglial activation mediates tolerance to the neurotoxic effects of methamphetamine. J. Neurochem., 92, 790-797, 2005.

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Sakowski, S.A., Geddes, T.J. and **Kuhn, D.M.** Mouse tryptophan hydroxylase isoform 2 and the role of proline 447 in enzyme function. J. Neurochem., 96, 758-765, 2006.

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Kuhn, D.M., Sakowski, S.A., Geddes, T.J., Wilkerson, C., and Haycock, J.W. Phosphorylation and activation of tryptophan hydroxylase 2: Identification of serine-19 as the substrate site for calcium-dependent protein kinase II. J. Neurochem., 103, 1567-1573, 2007.

Thomas, D.M., Francescutti-Verbeem, D.M., and **Kuhn, D.M.** The newly synthesized pool of dopamine determines the severity of methamphetamine-induced neurotoxicity. J. Neurochem., 605-616, 2008.

Kuhn, D.M., Francescutti-Verbeem, D.M., and Thomas, D.M. Dopamine disposition in the presynaptic process regulates the severity of methamphetamine-induced neurotoxicity. Ann. N.Y. Acad. Sci., in press, 2008.

Ni, W., Geddes, T.J., Priestley, J.R., Szasz, T., **Kuhn, D.M.**, and Watts, S.W. The existence of a local serotonergic system in rat peripheral arteries. Brit. J. Pharmacol., in press, 2008.

Linder, A.E., Ni, W., Szasz, T., Burnett, R., Diaz, J., Geddes, T.J., **Kuhn, D.M.**, and Watts, S.W. A serotonergic system in veins: serotonin-transporter independent uptake. J. Pharmacol. Exp. Ther., in press, 2008.

Thomas, D.M., Francescutti-Verbeem, D.M., and **Kuhn, D.M.** Methamphetamine-induced neurotoxicity and microglial activation are not mediated by fractalkine receptor signaling. J. Neurochem. in press, 2008.

Principal Investigator/Program Director (Last, First, Middle): Kuhn, Donald M.

Research Support

Ongoing (Active) Research Support

NIH/NIDA 5 R01 DA10756 04/10/07-04/09/12
Neurotoxic Amphetamines, Radicals, and 5HT Neurons
The major goal of the study is to determine the mechanisms by which neurotoxic amphetamine-derived reactive oxygen and nitrogen species alter function of dopamine and serotonin neurons through their effects on important phenotypic marker proteins in these neuronal elements.
Role: PI

NIH/NIDA 1 RO1 DA017327 04/01/05 – 03/30/10
Methamphetamine Neurotoxicity and Microglial Activation
The goal of this project is to elucidate the role of microglia in the neurotoxic effects associated with methamphetamine and other neurotoxic amphetamines. When funded, the budget of DA017327 was reduced by 25% (arbitrary) and the decision of which projects could be completed was left to the discretion of the PI. Because this area (neurotoxic amphetamines/microglia) has received considerable interest in our lab and in a growing number of others, and considering that this represents nearly the entire thrust of our lab, the some of the studies that had to be cut from DA017327 are now part of DA10756 (this application).
Role: PI

Department of Veterans Affairs Merit Award 03/15/07-03/14/11
Brain Injury by Blast Overpressure: Role of Microglial Activation
The goal of this project is to characterize microglial involvement in brain damage caused by blast overpressure. We have developed a model of blast overpressure, a form of traumatic brain injury, that allows testing of cultured cells and brain slices.
Role: PI

Projects completed in the past 3 years

NIH/NIDA 1 K05 DA14692 10/05/02-12/04/07
Molecular Biology of Drug Abuse
This is a senior scientist career development award.
Role: PI

NIH/NIDA 1 T32 DA07310 07/01/00-06/30/06
Neuroscience Training in Drug Abuse
This is a training grant that supports two predoctoral and two postdoctoral fellows. This training program is in hiatus temporarily. Our department experienced some significant changes in faculty re-assignment to other academic units, and several other key investigators on the T32 have left Wayne State. Therefore, we are re-configuring this training program as the Translational Neuroscience Program to reflect more accurately the current mentoring and research expertise of our departmental faculty. We anticipate submission of a renewal application in March 2007.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Mueller, Patrick J.	Assistant Professor
eRA COMMONS USER NAME MUELLERP	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Blackburn University, Carlinville, Illinois	B.A.	1990	Biology/Chemistry
St. Louis University, St. Louis, Missouri	Ph.D.	1996	Pharmacol. & Physiol. Sci.
Medical College of Wisconsin, Milwaukee, WI	Postdoc	1995-1997	Exercise Phys./Neural Ctrl.
University of Missouri, Columbia, MO	Postdoc	1997-2001	Neural Ctrl. of Circulation

NOTE: The Biographical Sketch may not exceed four pages. Follow the formats and instructions on the attached sample.

A. Positions and Honors.

Positions and Employment

1990-1995	Graduate Trainee, St. Louis University Health Sciences Center
1995-1997	Postdoctoral Fellow, Medical College of Wisconsin
1997-2001	Postdoctoral Fellow, University of Missouri-Columbia (MU)
2001-2/2007	Research Assistant Professor, Dalton Cardiovascular Research Center, MU
2003-2/2007	Research Investigator, Dalton Cardiovascular Research Center, MU
2003-2/2007	Adjunct Research Assistant Professor, Department of Biomedical Sciences, MU
3/2007-	Assistant Professor, Department of Physiology, Wayne State University
8/2007-	Graduate Faculty, , Wayne State University

Honors

1989	Bonnie Keith Albracht Scholarship, Blackburn College, Carlinville IL
1989	C.H.C. Anderson Prize, Blackburn College, Carlinville IL
1989	Drew Thurston Memorial Award, Blackburn College, Carlinville IL
2001	Caroline tum Suden/Frances A. Hellebrandt Professional Opportunity Award, American Physiological Society, Experimental Biology Meeting
2001	Michael J. Brody Young Investigator Award, APS Neural Control and Autonomic Regulation Section, Experimental Biology Meeting
2001	Phi Zeta Research Day, 1st Place Oral Presentation, Advanced Graduate Students and Postdocs
2005	Research Recognition Award, APS Neural Control and Autonomic Regulation Section, Experimental Biology Meeting
2006	Research Career Enhancement Award, American Physiological Society Host Laboratory: Patrice Guyenet, Ph.D., University of Virginia
2007	Outstanding Poster picked for Oral Presentation FASEB Summer Research Conference: Sydney, Australia Neural Mechanisms in Cardiovascular Regulation

Other Experience and Professional Memberships

Reviewer: Am J Physiol: Heart Circ Physiol; Am J Physiol: Reg Integr Physiol; J Appl Physiol; Med Sci Sports & Exercise, Hypertension, Exp Physiol, BMC Neurosci. University of Florida Mock Grant Review (03/2004), American Heart Association National Consortium Peer Review Committee (10/07).
Societies: American Physiological Society, Society for Neuroscience, American College of Sports Medicine

Related Activities: Faculty Grant Writing Institute-University of Missouri-Columbia (05/06); Lecturer, Univ. of Missouri-Columbia, Biomed. Sci. Course VBSCI9467 "Neural Control of the Circulation" (Spring '02,'04,'06); Instructor, Central Neural Control of the Circulation, The American Physiological Society Latin American Initiative, Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo-Brazil (08/04); Poster Judge, Cardiovascular Day, Columbia, MO (02/04); Architect Advisory Committee-Dalton Cardiovascular Research Center Expansion and Renovation Project (2001-2003); APS Committee Member, Neural Control and Autonomic Regulation Steering Committee, Member in Training (2001-2002); Lecturer, Wayne State University School of Medicine, Dept of Physiol PSL7600 "Advanced Cardiovascular Physiology" (Spring '02,'04,'06); Laboratory Instructor, Medical Physiology, Wayne State University School of Medicine.

B. Peer-reviewed publications.

1. Knuepfer, M.M., Branch, C.A., **Mueller, P.J.** and Gan, Q. Stress and cocaine elicit similar cardiac output responses in individual rats. Am J Physiol 265 (Heart Circ. Physiol. 34): H779-82, 1993.
2. **Mueller, P.J**. and Knuepfer, M.M. Coronary vascular effects of cocaine in rats. J Pharmacol Exp Ther 268: 97-103, 1994.
3. **Mueller, P.J.**, Gan, Q. and Knuepfer, M.M. Ethanol alters hemodynamic responses to cocaine in rats. *Drug Alcohol Depend* 48:17-24, 1997.
4. Buckwalter, J.B., **Mueller, P.J.** and Clifford, P.S. Autonomic control of skeletal muscle vasodilation during exercise. *J Appl Physiol* 83 (6): 2037-2042, 1997.
5. Buckwalter, J.B., **Mueller, P.J.** and Clifford, P.S. Sympathetic vasoconstriction to active skeletal muscles during dynamic exercise. *J Appl Physiol* 83 (5):1575-80, 1997.
6. Buckwalter, J.B., **Mueller, P.J.** and Clifford, P.S. α_1-Adrenergic receptor responsiveness in skeletal muscle during dynamic exercise. *J. Appl. Physiol.* 85 (6): 2277-2283, 1998.
7. Buckwalter, J.B., Ruble, S.B., **Mueller, P.J.** and Clifford, P.S. Skeletal muscle vasodilation at the onset of exercise. *J Appl Physiol* 85 (5): 1649-1654, 1998.
8. **Mueller, P.J.**, O'Hagan, K.P., Skogg, K.A., Buckwalter, J.B. and Clifford, P.S. Renal hemodynamic responses to dynamic exercise in rabbits. *J Appl Physiol* 85 (5): 1605-1614, 1998.
9. Knuepfer, M.M., Gan, Q. and **Mueller, P.J.** Mechanisms of hemodynamic responses to cocaine in conscious rats. *J Cardiovasc Pharmacol* 31:391-399, 1998.
10. Knuepfer, M.M. and **Mueller, P.J.** Review of evidence for a novel model of cocaine-induced cardiovascular toxicity. *Pharmacol. Biochem. Behav.* 63 (3): 489-500, 1999.
11. Foley, C.M., Vogl, H.W., **Mueller, P.J.**, Hay, M. and Hasser, E.M. Cardiovascular response to group I metabotropic glutamate receptor activation in nucleus tractus solitarius. *Am. J. Physiol.* 276 (*Regulatory Integrative Comp. Physiol.* 45): R1469-R1478, 1999.
12. Coon, R.L., **Mueller, P.J.** and Clifford, P.S. Functional anatomy of the vagal innervation of the cervical trachea of the dog. *J. Appl. Physiol.* 89(1):139-42, 2000.
13. **Mueller, P.J.**, Cunningham, J.T., Patel, K.P. and Hasser, E.M. Proposed role of the paraventricular nucleus in cardiovascular deconditioning. *Acta Physiol Scand* 177:27-35, 2003.
14. **Mueller, P.J.** and Hasser, E.M. Enhanced sympathoinhibitory response to volume expansion in conscious hindlimb unloaded rats. *J. Appl. Physiol.* 94: 1806-1812, 2003.
15. **Mueller, P.J.**, Buckwalter, J.B. and Clifford, P.S. Tracheal tone and the role of ionotropic glutamate receptors in the nucleus ambiguus. *Brain Research* 1021: 54-62, 2004.
16. **Mueller, P.J.**, Foley, C.M., and Hasser, E.M. Hindlimb unloading alters nitric oxide and autonomic control of resting arterial pressure in conscious rats. Am. J. Physiol. Reg. Integr. Comp. Physiol. 289: R140-R147, 2005.
17. **Mueller, P.J.**, Foley, C.M., Vogl, H.W., Hay, M. and Hasser, E.M. Cardiovascular response to group III mGluR activation in NTS requires NMDA receptors. Am. J. Physiol. Reg. Integr. Comp. Physiol. 289: R198-R208, 2005.
18. Foley, C.M., **Mueller, P.J.**, Hasser, E.M. and Heesch, C.M. Hindlimb unloading and female gender attenuate baroreflex mediated sympathoexcitation. Am. J. Physiol. Reg. Integr. Comp. Physiol. 289: R1440-R1447, 2005.
19. **Mueller, P.J.** and Hasser, E.M. Putative role of NTS in alterations in neural control of the circulation following exercise training in rats. Am. J. Physiol. Reg. Integr. Comp. Physiol. 290: R383-R392, 2006.

B. Peer-reviewed publications (cont.)

20. **Mueller, P.J.**, Sullivan, M.J., Grindstaff, R.R., Cunningham, T.J. and Hasser, E.M. Regulation of plasma vasopressin and renin activity in conscious hindlimb-unloaded rats. Am. J. Physiol. Reg. Integr. Comp. Physiol. 291: R46-R52, 2006.
21. **Mueller, P.J.**, Foley, C.M., Heesch, C.M. Cunningham, T.J., Zheng, H., Patel, K.P. and Hasser, E.M. Increased nitric oxide synthase in the hypothalamus of hindlimb unloaded rats. Brain Res. 1115: 65-74, 2006.
22. **Mueller, P.J.** Exercise training attenuates increases in lumbar sympathetic nerve activity produced by stimulation of the rostral ventrolateral medulla. J. Appl. Physiol. 102(2):803-13, 2007.
23. **Mueller, P.J.** Exercise training and sympathetic nervous system activity: Evidence for physical activity dependent plasticity. J. Clin. Exp. Pharmacol. Physiol. 34(4):377-84, 2007.

C. Research Support

Ongoing Research Support

HL089364 08/10/07-06/30/09
R21, National Institutes of Health
"Physical Activity Dependent Plasticity in Central Sympathetic Nervous System Regulation"
The major goals of this project are to 1) Determine the extent to which physical inactivity affects the excitability of spinally projecting RVLM neurons. 2) Determine the extent to which physical inactivity affects the structure of spinally projecting RVLM neurons.
Role: PI

R21, National Institutes of Health (HL089364) Supplement 1 08/10/07-06/30/09
"Physical Activity Dependent Plasticity in Central Sympathetic Nervous System Regulation"
This supplement supports an undergraduate minority student working in my laboratory- Janet Adedokun

R21, National Institutes of Health (HL089364) Supplement 2 08/10/07-06/30/09
"Physical Activity Dependent Plasticity in Central Sympathetic Nervous System Regulation"
This supplement supports an undergraduate minority student working in my laboratory- Jason Franco

0650161Z*
Grant In Aid, American Heart Association, Heartland Affiliate 01/01/06-12/31/07
Central Control of Sympathetic Outflow Following Exercise Training
The major goals of this project are to 1) Examine the effect of ExTr on activation of SNS activity and spinally projecting RVLM neurons. 2) Examine the effect of ExTr on regulation of SNS outflow by altering tonic excitatory and inhibitory neurotransmission in the RVLM.
Role: PI

*Transferred to Wayne State University from University of Missouri 03-01-07, under no cost extension for 2008

Pending Research Support

Grant In Aid, American Heart Association, Heartland Affiliate 07/08-06/10
"Physical Inactivity/Activity Induced Alterations in Sensitivity of Sympathoexcitatory Neurons in the RVLM" (Pending)
Role: Principal Investigator

Completed Research Support

National Institutes of Health Predoctoral Training Fellow 1991-1993
Department of Pharmacological and Physiological Science
St. Louis University Health Sciences Center
Role: Graduate Research Assistant

Postdoctoral Fellowship, American Heart Association 07/98-03/99*
"Mechanisms of Area Postrema Sympathoinhibition"
*Returned 03/99 for NRSA awarded in 04/99
Role: Postdoctoral Fellow

HL10166-01
National Research Service Award (F32), National Institutes of Health 04/99-03/02
"Mechanisms of Area Postrema Sympathoinhibition"
This project examined the role of the area postrema in regulating sympathetic outflow in conscious and anesthetized rats.
Role: Postdoctoral Fellow

0265264Z 07/01/02-6/30/04
Beginning Grant in Aid, American Heart Association, Heartland Affiliate
Central Autonomic Regulation Following Exercise
The major goals of this project are to 1) examine the effect of exercise training on neurohumoral control of circulation following hemorrhage 2) determine the role of cardiopulmonary receptor activation during hemorrhage following exercise training 3) Determine potential brain areas that are responsible for altered neurohumoral control of the circulation following exercise training 4) determine whether altered neuronal activation in ET rats is due to enhanced inhibitory influence from cardiopulmonary receptors.
Role: PI

HL55306 04/01/97-3/30/02
R01, National Institutes of Health
Cardiovascular Regulation in Hindlimb Unweighted Rats
The major goals of this project are to 1) evaluate arterial and cardiopulmonary baroreflex regulation of renal and lumbar sympathetic nerve activity in conscious rats subjected to 14 days of hindlimb unloading; 2) examine the interaction between arterial and cardiac baroreflex control of sympathetic nerve activity in conscious hindlimb unweighted rats; 3) evaluate changes in afferent and/or central nervous system mechanisms in baroreflex regulation of the sympathetic nervous system; and 4) evaluate changes in vasomotor reactivity of visceral and skeletal muscle vascular beds in conscious rats.
Role: Postdoctoral Fellow
PI: Eileen M. Hasser Ph.D.

HL55306 04/01/02-03/31/06
R01, National Institutes of Health
Cardiovascular Regulation in Hindlimb Unweighted Rats
The major goals of this project are to 1) evaluate arterial and cardiopulmonary baroreflex regulation of renal and lumbar sympathetic nerve activity in conscious rats subjected to 14 days of hindlimb unloading; 2) examine the interaction between arterial and cardiac baroreflex control of sympathetic nerve activity in conscious hindlimb unweighted rats; 3) evaluate changes in afferent and/or central nervous system mechanisms in baroreflex regulation of the sympathetic nervous system; and 4) evaluate changes in vasomotor reactivity of visceral and skeletal muscle vascular beds in conscious rats.
Role: Co-Investigator
PI: Eileen M. Hasser Ph.D.

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian, W

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
E. Mark Haacke, Ph.D.	Director
eRA COMMONS USER NAME	
ak5444	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Toronto	B.S.	1973	Mathematics & Physics
University of Toronto	M.S.	1975	Theoretical Physics
University of Toronto	Ph.D.	1978	High Energy Physics

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors.

POSITIONS

1981-1983 **Research Geophysicist**, projects seismic tomography, scattering theory and imaging, Gulf Research and Development, Pittsburgh, PA

1983-1985 **Senior Research Scientist**, projects included NMR sequence development, fast imaging, reduction of motion artifacts, chemical shift imaging, new reconstruction schemes, Picker International, Highland Heights, OH

1983-1985 **Lecturer in Physics**, developed a new course on MRI, Case Western Reserve University, Cleveland, OH

1985-1989 **Assistant Professor of Radiology and Physics**, Head, MR Physics and Basic Science. Developed flow, motion and fast imaging techniques as well as new high resolution, high S/N reconstruction techniques, Case Western Reserve University

1989-1993 **Associate Professor**, in the Department of Radiology with joint appointments in Physics and Biomedical Engineering, Case Western Reserve University

1993-1999 **Professor of Radiology**, Director MR Imaging Research, Mallinckrodt Institute of Radiology, Washington University, St. Louis, MO

1999-present **Director**, The Magnetic Resonance Imaging Institute for Biomedical Research, St. Louis, MO

2002-present **Professor of Radiology**, Wayne State University, Detroit, MI

HONORS

1975 Ontario Graduate Scholarship

1976 Ontario Graduate Scholarship

1977 E.F. Burton Fellowship

1989 Sylvia Sorken Greenfield Award for the best paper in Medical Physics

1992 Fellow of the Society Award for the Society of Magnetic Resonance Imaging

1994 Silver Medal Award, Society of Magnetic Resonance

1997 Poster Award at the 14th Annual Meeting, European Society for Magnetic Resonance in Medicine and Biology. J.R. Reichenbach, E.M. Haacke, B.C.P. Lee, Ch. Przetak, W.A. Kaiser

1998 Marie-Sklodowska-Curie Prize for Visualization of Cerebral Venous Structures Using High Resolution MRI by J.R. Reichenbach, L.R. Schad, M. Essig, E.M. Haacke, W.A. Kaiser

2000 Poster Prize of the XXVI Congress of the European Society of Neuroradiology 2000. J.R. Reichenbach, L. Jonetz-Mentzel, C. Fitzek, H.-J. Mentzel, E.M. Haacke, W.A. Kaiser.

2002 Scientific Exhibition Award ECR 2002 Cum Laude. J.R. Reichenbach, C. Fitzek, L. Jonetz-Mentzel, D. Sauner, H.-J. Mentzel, E.M. Haacke, W.A. Kaiser. European Congress of Radiology

2004 Gold Medal Award, International Society of Magnetic Resonance in Medicine

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian, W

B. Selected peer-reviewed publications (in chronological order).
(Publications selected from 163 peer-reviewed publications)

1. F.G.C. Hoogenraad, P.J.W. Pouwels, M.B.M. Hofman, S.A.R.B. Rombouts, C. Lavini, M.O. Leach, E.M. Haacke. High-resolution Segmented EPI in a Motor Task fMRI Study. Magnetic Resonance Imaging 2000; 18:405-409.
2. E.H. Haacke, Z-P. Liang. Challenges of Imaging Structure and Function with MRI. IEEE Eng Med Biol 2000;19(5):55-62.
3. Y. Wang, Y. Yu, D. Li, K.T. Bae, J.J. Brown, W. Lin, E.M. Haacke. Artery and Vein Separation Using Susceptibility Dependent Phase in Contrast-Enhanced MRA. JMRI 2000; 12:661-670.
4. J.R. Reichenbach, M. Barth, E.M. Haacke, M. Klarhöfer, W.A. Kaiser, E. Moser. High-Resolution MR Venography at 3.0 Tesla. J Comp Assist Tomog 2000; 24(6): 949-957.
5. F.G.C. Hoogenraad, P.J. W. Pouwels, M.B.M. Hofman, J.R. Reichenbach. M. Sprenger, E.M. Haacke. Quantitative Differentiation Between BOLD Models in fMRI. MRM 2001; 45:233-246.
6. Y-C.N. Cheng, E.M. Haacke, Y-J. Vu. An Exact Form for the Magnetic Field Density of States for a Dipole. MRI 2001; 19:1017-1023.
7. D. Li, J. Zheng, H.J. Weinmann, K.T. Bae, E.M. Haacke. Contrast-enhanced MRI of Coronary Arteries: Comparison of Intravascular and Extravascular Contrast Agents and Technical Consideration. Radiology 2001; 218:670-678.
8. K. Scheffler, E. Seifritz, D. Bilecen, R. Venkatesan, J. Hennig, M. Deimling, E.M. Haacke. Detection of BOLD Changes by Means of a Frequency-sensitive TrueFISP Technique: Preliminary Results. NMR in Biomedicine 2001; 14:490-496.
9. Yu-Chung N. Cheng, E. Mark Haacke. Predicting BOLD signal changes as a function of blood volume fraction and resolution. NMR in Biomedicine 2001;14:468-477.
10. K. Gurleyik, E. Mark Haacke. Quantification of Errors in Volume Measurements of the Caudate Nucleus Using Magnetic Resonance Imaging. JMRI 2002;15:353-363.
11. E.M. Haacke, G. Herigault, Y. Yu, D. Kido, K. Tong, A. Obenaus, J. Reichenbach. Observing Tumor Vascularity Noninvasively Using Magnetic Resonance Imaging. Image Anal Stereol 2002;21:107-113.
12. Tong, K.A., Ashwal, S., Holshouser, B.A., Shutter, L., Herigault, G., Haacke, E.M., Kido, D.K. Hemorrhagic Shearing Lesions in Children and Adolescents with Posttraumatic Diffuse Axonal Injury: Improved Detection and Initial Results. Radiology 2003; 227:332–339.
13. Tong, K.A., Ashwal, S., Holshouser, B.A., Shutter, L., Herigault, G., Haacke, E.M., Kido, D.K. Improved Detection of Hemorrhagic Shearing Lesions in Children with Post-traumatic Diffuse Axonal Injury – In Children: Clinical Correlation with Hemorrhagic Lesions. Ann Neurol 2004;56:36–50.
14. N.D. Wycliffe, J. Choe, B. Holshouser, U.E. Oyoyo, E.M. Haacke and D. Kido. Reliability in Detection of Hemorrhage in Acute Stroke by a New Three-Dimensional Gradient Recalled Echo Susceptibility-Weighted Imaging Technique Compared to Computed Tomography: A Retrospective Study. JMRI 2004;20:372-377.
15. R.K. Singh, V.S., Deshpande, E.M. Haacke, S.M. Shea, Y. Xu, R.M. McCarthy, J. Carr, and D. Li. Coronary Artery Imaging Using Three-Dimensional Breath-Hold Steady-State Free Precession with Two-Dimensional Iterative Partial Fourier Reconstruction. JMRI 2004;19:645-649.
16. E.M. Haacke, Y. Xu, Y.C.N. Cheng, J. Reichenbach. Susceptibility Weighted Imaging (SWI). MRM 2004;52:612-618.
17. D. Haddar, E.M. Haacke, V. Sehgal, Z. DelProposto, G. Salamon, O. Seror, N. Sellier. L'imagerie de susceptibilite magnetique: theorie et applications. J Radiol 2004;85;1901-1908.
18. A. Ruascher, J. Sedlacik, M. Barth, E.M. Haacke, J.R. Reichenbach. Non-invasive Assessment of Vascular Architecture and During Modulated Blood Oxygenation Using Susceptibility Weighted MRI (SWI). (In review at MRM – Dec 2004).
19. V. Sehgal, Z. Delproposto, D. Haddar, E.M. Haacke, A.E. Sloan, L.J. Zamorano, G. Barger, J. Hu, Y. Xu, K.P. Prabhakaran, I.R. Elangovan, J. Neelavalli, J.R. Reichenbach. Susceptibility Weighted Imaging of Brain Masses. (In review at JMRI – Dec 2004).
20. E.M. Haacke, N.Y.C. Cheng, M.J. House, Q. Liu, J. Neelavalli, R.J. Ogg, A. Khan, M. Ayaz, W. Kirsch, A. Obenaus. Imaging Iron Store in the Brain Using Magnetic Resonance Imaging. MRI 2005; 23;1–25.
21. J. Hu, Y. Xia, W. Feng, Y. Xuan, Y. Shen, E. M. Haacke, Q. Jiang. Orientational dependence of trimethyl ammonium signal in human muscles by 1H magnetic resonance spectroscopic imaging. MRI 2005; 23;97–104.

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian, W

22. Shen Y, Kou Z, Kreipke CW, Petrov T, Hu J, Haacke EM. 2006. In vivo measurement of tissue damage, oxygen saturation changes and blood flow changes after experimental traumatic brain injury in rats using susceptibility-weighted imaging. Magn Reson Imaging 25(2):219-227.

C. Research Support.

1. R01 AG20948 Kirsch, Wolff – PI (Subcontract with Loma Linda University, California)
   BRP 10/1/2002-9/30/2007
   Iron Metabolism Alteration in Alzheimer 's Disease

   Goals: Evaluate the role of IRP-2 in Alzheimer'sdisease and the role of a new MRI technique to image brain iron. Role: Co-Investigator

2. Siemens Medical Solutions Haacke, E.Mark – PI
   Master Research Agreement
   7/1/01 – 6/30/06

   Goals: Collect clinical data for SWI in the areas of trauma, stroke, and vascular disease. Role: PI

3. R01 NS038292 Jiang, Quan – PI (Subcontract with Henry Ford Hospital, Michigan)
   NINDS 07/01/04 – 06/30/08
   MRI and SVZ Cell Therapy for Severe Stroke

   Goals: Develop noninvasive in vivo MRI methodology for tracking transplanted MSCs and their effects on the host brain. Role: Co-Investigator

Ongoing Research Support for Dr. Kreipke

R01 NS39860 J Rafols (PI) 3/10/04-4/30/09
NIH-NINDS
Role: CO-I (70% effort)
"Control of microvascular tone in traumatic brain injury"
Investigates the role of endothelin receptors in the control of the microcirculation in a rat model of traumatic brain injury.

VA RR&D Award 1/01/08-12/31/11
VA Rehabilitation Award
Role: CO-I (30% effort)
"Conditioning, microvascular tone & rehabilitation"
Investigates the role of exercise in controlling microcirculation after traumatic brain injury.

Ongoing Research Support for Dr. Rafols

NIH-NINDS RO1 NS39860 (PI) 06/01/04-05/31/09
Control of Microvascular tone in Traumatic Brain Injury.
Co-I

NIH-NINDS RO1 NS044100 (CO-I) 07/01/03-06/30/08
The Unfolded Protein Response after Brain Ischemia.
Co-I

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian, W.

Research Support for Dr. Kuhn

Ongoing (Active) Research Support

NIH/NIDA 1 K05 DA14692 10/05/02-10/04/08
Molecular Biology of Drug Abuse
This is a senior scientist career development award
Role: PI

NIH/NIDA 1 RO1 DA017327-01 04/01/05 – 03/30/10
Methamphetamine Neurotoxicity and Microglial Activation
The goal of this project is to elucidate the role of microglia in the neurotoxic effects associated with methamphetamine and other neurotoxic amphetamines.
Role: PI

Research Support for Dr. Haacke.

1. R01 AG20948 Kirsch, Wolff – PI (Subcontract with Loma Linda University, California)
   BRP 10/1/2002-9/30/2008
   Iron Metabolism Alteration in Alzheimer 's Disease
   Role: Co-Investigator
   Goals: Evaluate the role of IRP-2 in Alzheimer's disease and the role of a new MRI technique to image brain iron.

2. R01 NS038292 Jiang, Quan – PI (Subcontract with Henry Ford Hospital, Michigan)
   NINDS 07/01/04 – 06/30/08
   MRI and SVZ Cell Therapy for Severe Stroke
   Role: Co-Investigator
   Goals: Develop noninvasive in vivo MRI methodology for tracking transplanted MSCs and their effects on the host brain.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix: Dr.
* First Name: Christian
Middle Name: William
* Last Name: Kreipke
Suffix: Ph.D.

* New Investigator? ☐ No ☒ Yes

Degrees: PhD

2. Human Subjects

Clinical Trial? ☒ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix: Ms.
* First Name: Carole
Middle Name:
* Last Name: Bach
Suffix:

* Phone Number: 313.577.2294
Fax Number: 313.577.2653
Email: orpsmail@wayne.edu

* Title: Director, Pre-Award Services

* Street1: 4201 St. Antoine, 9D-UHC
Street2:
* City: Detroit
County:
* State: MI: Michigan
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 48201

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

PHS 398 Modular Budget, Periods 1 and 2

OMB Number: 0925-0001

Budget Period: 1

Reset Entries | Start Date: 11/01/2009 | End Date: 10/31/2010

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Modified Total Indirect Costs	52		130,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): U.S. Department of Education, Richard Dowd, 312-886-6503

Indirect Cost Rate Agreement Date: 08/25/2008

Total Indirect Costs: 130,000.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 380,000.00

Budget Period: 2

Reset Entries | Start Date: 11/01/2010 | End Date: 10/31/2011

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Modified Total Indirect Costs	52		130,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): U.S. Department of Education, Richard Dowd, 312-886-6503

Indirect Cost Rate Agreement Date: 08/25/2008

Total Indirect Costs: 130,000.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 380,000.00

PHS 398 Modular Budget, Periods 3 and 4

Budget Period: 3

Reset Entries | Start Date: 11/01/2011 | End Date: 10/31/2012

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Modified total indirect costs	52		130,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): U.S. Department of Education, Richard Dowd, 312-886-6503

Indirect Cost Rate Agreement Date: 08/25/2008

Total Indirect Costs: 130,000.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 380,000.00

Budget Period: 4

Reset Entries | Start Date: 11/01/2012 | End Date: 10/31/2013

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Modified total indirect costs	52		130,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): U.S. Department of Education, Richard Dowd, 312-886-6503

Indirect Cost Rate Agreement Date: 08/25/2008

Total Indirect Costs: 130,000.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 380,000.00

PHS 398 Modular Budget, Periods 5 and Cumulative

Budget Period: 5

Reset Entries | Start Date: 11/01/2013 | End Date: 10/31/2014

A. Direct Costs

	* Funds Requested ($)
* Direct Cost less Consortium F&A	250,000.00
Consortium F&A	
* Total Direct Costs	250,000.00

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	* Funds Requested ($)
1.	Modified total indirect costs	52		130,000.00
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): U.S. Department of Education, Richard Dowd, 312-886-6503

Indirect Cost Rate Agreement Date: 08/25/2008

Total Indirect Costs: 130,000.00

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): 380,000.00

Cumulative Budget Information

1. Total Costs, Entire Project Period

*Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$	1,250,000.00
Section A, Total Consortium F&A for Entire Project Period	$	
*Section A, Total Direct Costs for Entire Project Period	$	1,250,000.00
*Section B, Total Indirect Costs for Entire Project Period	$	650,000.00
*Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$	1,900,000.00

2. Budget Justifications

Personnel Justification	1255-Personnel.pdf	Add Attachment	Delete Attachment	View Attachment
Consortium Justification		Add Attachment	Delete Attachment	View Attachment
Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

Personnel

Christian Kreipke, Ph.D., Principal Investigator (7.2 cal. mos.) is an emerging investigator in the field of TBI research. He joined Dr. Rafols' laboratory to conduct brain trauma research over three years ago and, hence has finished his post-doctoral training. During the current lab funding, he has enhanced the laboratory's goals by including behavioral analysis and added to the pharmacological experiments designed. Dr. Kreipke has experience in mentoring students, working with the model of brain injury, molecular biology and in assessing animal behavior. He will oversee the overall completion of the work and will facilitate weekly meetings of the investigators to discuss findings. In conjunction, Dr. Kreipke will personally conduct many of the experiments and will oversee training of student assistants. He will be reduced to 30% on Dr. Rafols current RO1 and 10% on Dr. Rossi's VA RR&D Award. His resposibilities will be facilitated by a new post-doctoral fellow in both laboratories. *Note: This change was made from the first submission because it was felt by all investigators that Dr. Kreipke should focus the majority of his time on his own proposal.*

Jose Rafols, Ph.D., CO-Investigator (1.8 cal. mos.) has over thirty years of experience performing studies of brain ischemia and TBI. He will assist Dr. Kreipke in facilitating the reaserch design. In addition, he will aide in the final preparation of mansucripts and grants that directly accompany this work.

Donald Kuhn, Ph.D., (1.8 cal. mos.) is an expert in molecular biology and pharmacology and will provide expertise and access to Western protocols and will assist in pharmacological treatments (see letter of support).

Patrick Mueller, Ph.D., (1.2 cal. mos.) is an expert in vascular physiology and will be instrumental in carrying out all systemic heart rate and blood pressure measurements. He will also aide in the final preparation of mansucripts pertaining to systemic effects of drug treatments.

E. Mark Haacke, Ph.D., (consultant) is a leading authority in neuroimaging. While Dr. Kreipke will physically perform all neuroimading scans, Dr, Haacke will be available for consultation regarding acquisition and interpretation of all data requiring magnetic resonance imaging.

Patrick Schafer, Research technician (6 cal. mos.) will be instrumental in carrying out immunocytochemistry and Western analysis. He is also trained in surgery and behavioral analysis. He has currently published in the laboratory. Further, one initiative within this proposal is the training of students in neurosciences. This training will be overseen by Dr. Kreipke.

Steven Schafer, Research technician (6 cal. mos.) will be instrumental in carrying out the behavioral analysis. As evidenced in the proposal, much of our behavioral work is done 20-30 days consecutively and is conducted during evening hours to correspond with the active cycle of rat's diurnal cycle. Therefore, we feel a dedicated person to this task who will not have to conduct experiments during day hours is needed. Further, one initiative within this proposal is the training of students in neurosciences. This training will be overseen by Dr. Kreipke.

Close Form | Print Page | About

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page and PHS398 Checklist. The responses provided on these pages, regarding the type of application being submitted, are repeated for your reference, as you attach the appropriate sections of the research plan.

*Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)	1240-Response to Reviewers A	Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	1241-SPECIFIC AIMS REVISED A	Add Attachment	Delete Attachment	View Attachment
3. Background and Significance	1242-BACKGROUND AND SIGNIFI	Add Attachment	Delete Attachment	View Attachment
4. Preliminary Studies / Progress Report	1243-PRELIMINARY DATA REVISE	Add Attachment	Delete Attachment	View Attachment
5. Research Design and Methods	1244-RESEARCH DESIGN AND ME	Add Attachment	Delete Attachment	View Attachment
6. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
7. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

Attachments 8-11 apply only when you have answered "yes" to the question "are human subjects involved" on the R&R Other Project Information Form. In this case, attachments 8-11 may be required, and you are encouraged to consult the Application guide instructions and/or the specific Funding Opportunity Announcement to determine which sections must be submitted with this application.

8. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
10. Targeted/Planned Enrollment		Add Attachment	Delete Attachment	View Attachment
11. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

12. Vertebrate Animals		Add Attachment	Delete Attachment	View Attachment
13. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
14. Multiple PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
15. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
16. Letters of Support		Add Attachment	Delete Attachment	View Attachment
17. Resource Sharing Plan(s)		Add Attachment	Delete Attachment	View Attachment

18. Appendix Add Attachments | Remove Attachments | View Attachments

General comment: Once again I would like to thank the review panel for their enthusiasm and encouragement in pursuing this project. As a new investigator and an Early Stage Investigator (ESI) I am delighted by the overall positive review of the A1 proposal. In this A2 and final revision I have endeavored to effectively address the requested points which need further clarification. Each critique was carefully reviewed and responded to either in text or with new preliminary data. In so doing the scientific merit of the proposal has been strengthened, hopefully bringing it to funding levels.

NOTE: All changes are indicated by *italics*.

Overall Comment: There were three outstanding issues, each articulated by a different reviewer. Therefore I will move directly to address these issues under each of the individual reviewers.

Reviewer 1.

Comment: "The issue which still remains unclear is how reduced CBF causes additional injury to the brain when it is injured".

Address: A goal of our ongoing and future work is to answer this issue. In fact, as pointed out at the 2008 Neurotrauma Meeting in Orlando, FL, the interaction/inter-relatedness of multiple pathologies and the respective contribution to secondary injury represent a large gap in the field's knowledge. As our preliminary data have determined, TBI causes neurons to be vulnerable to relatively mild decreases in blood flow. Initial injury including diffuse axonal injury (DAI) causes the neurons to be compromised. This initial insult is then likely exacerbated by a significant decrease in metabolite delivery/availability and hypoxic conditions caused, in part, by the reduced blood flow. This combination of insults without doubt worsens histopathologic and behavioral outcomes. While important, such an investigation into the influence of multiple pathologies on neuronal integrity following TBI is felt to be beyond the scope of this proposal but will be studied in the future by our laboratory.

Reviewer 2.

Comment: "…in spatial distribution, how will an equivalent degree of CBF reduction by the phospho-Cp antibody be compared with the ETR antagonists—in which region are the levels equivalently blocked?"

Address: I apologize, here, if this was unclear. In actuality, the Cp-antibody was designed to ameliorate the hypoperfusion, not cause decreased blood flow. As reviewed in a recent publication in our laboratory (see appendix), phosphorylated Cp is removed from the acto-myosin complex which allows for cross-bridging and subsequent vasoconstriction. Therefore, by adding the antibody and competing out phosphorylated Cp we are aimed to block its disassociation from the acto-myosin complex, thus inhibiting vasoconstriction. As to the question of spatial distribution, I have made the preliminary data and research design more clear to reflect that blood flow measurements using the phospho-Cp antibody will be taken in the same regions (smCx and Hipp) as are taken for the ETr antagonist studies. Therefore, direct comparisons can be made and similar conclusions can be drawn. Furthermore, we will be prepared to do multiple injections of anti-Cp if necessary to maintain blood levels.

Reviewer 3.

Comment: It is apparent that the PI is anxious to pursue those studies [those pertaining to the clinically relevant ETrA antagonist] since his research plan for those experiments is pretty well thought out including an admirable consideration of dose-response. What is less clear, however, is whether he has adequately considered the possible need for repeating dosing over the 24-48 hr study period to maintain effective blood levels."

Address: We completely agree with the reviewer that, while our single-injection preliminary data seems to suggest that one injection is sufficient to ameliorate TBI-induced hypoperfusion, in order to most accurately mimic the clinical situation we should test a multi-injection approach in order to maintain blood flow levels throughout the duration of the experimental design (up to 48 hours post TBI). To that effect we are presently conducting this experiment using CBF measurements for outcome. In a very limited set of animals (N=3) we gave three injections of Clazosentan at 30 minutes, 24.5 and 48.5 hrs post TBI. CBF was maintained at or around control levels through the duration of the experiment. New preliminary is included as figure 14. Future experiments, if funded, will determine the behavioral effect after implementing the multiple-injection approach.

Minor Points:

Comment: It was felt that AIM 1 and possibly AIM 2 and 3 could be streamlined to accommodate the multiple-injection approach added to AIM 4.

Address: We agree and have condensed the work of AIM 1 into one-half year to make room for the expanded work in AIM 4.

Comment: It was felt that halothane may be a biohazard.

Address: We are aware of the potential hazard of using halothane, however in light of mounting evidence which suggests that isoflurane exhibits neuroprotective properties, we have chosen to remain with halothane. Given this, we have been in consultation with our IACUC and DLAR staff, and upon their guidance, we utilize not only a charcoal scavenging system, but also all experiments are conducted under a fume hood. With these precautions, we are fully approved by IACUC and our bio-safety office has deemed our experiments to be safe.

A. SPECIFIC AIMS

Traumatic brain injury (TBI) is reportedly the leading cause of death and disability among children and young adults (CDC Report, 2004). TBI is also known as the signature injury in the War on Terrorism. USA Today reported (2007) that 83% of brain injured marines and sailors returning from Iraq suffer lasting cognitive impairments. The combined emotional and financial costs of civilian casualties and those associated with the military as they try to rejoin civilian life is overwhelming. Therefore, there is a compelling need to implement effective therapies to improve the quality of life of those suffering from TBI.

Among multiple sequelae, TBI results in three major pathologies: 1) cerebral edema which leads to a critical rise in intracranial pressure, 2) diffuse axonal injury which brings about disruption of neural circuits underlying cognitive and motoric behaviors, and 3) alterations in the brain's microcirculation that cause a persistent state of hypoperfusion and improper delivery of vital metabolites to the brain parenchyma. Over 25 clinical trials aimed at the first two pathologies have been developed, none of which have been effective in the treatment for TBI (Povlishock, 2008). However to date no one has initiated a clinical trial addressing the third pathology, *dysfunctional vascular reactivity following TBI*. The present proposal provides rationale for proceeding towards a clinical trial by implementing novel strategies that aim to improve cerebral blood flow (CBF) and cognitive outcome following TBI. While our laboratory has published extensively on the role of endothelin-1 in mediating altered cerebral vascular reactivity after TBI, the cellular and molecular **mechanism** for this altered vasoreactivity remains to be elucidated. In addition **the causal relationship between ET-1, altered vasoreactivity and functional outcome has not been established**. This proposal addresses these issues by pharmacologic manipulation of the ET-1 system and calponin (Cp), a key element in vasoreactivity—the molecular events leading to vascular smooth muscle contractility and hence to vasoconstriction. **The central hypothesis of this proposal is: *TBI causes enhanced endothelin-1-mediated vasoconstriction and reduced CBF, which, in turn, exacerbates TBI-induced neuronal injury and cognitive deficits***. Four hypothesis-driven AIMS are designed to test this central hypothesis:

SPECIFIC AIM 1 tests the hypothesis that TBI causes enhanced ET-1 and endothelin receptors A and B (ETrA, B) expression which in turn, causes enhanced vasoconstriction and decreased CBF following TBI.

SPECIFIC AIM 2 tests the hypothesis that decreased CBF following TBI causes secondary neuronal injury following TBI.

SPECIFIC AIM 3 tests the hypothesis that a TBI-induced decrease in CBF leads to cognitive deficits via nerve cell injury.

SPECIFIC AIM 4 tests the hypothesis that IV injection of selective endothelin-A receptor antagonists, including Clazosentan, a new drug undergoing Phase II-III clinical trial for ameliorating vasospasm after stroke, and Sitaxesentan, a clinically approved drug, ameliorates TBI-induced decreases in CBF, improves neuronal histopathology and improves behavioral outcome following head trauma.

As a new investigator, the PI of this grant has assembled a team of experts that blend together the departments of anatomy, psychiatry, physiology, and radiology who will work closely with each other to investigate together the anatomical, molecular, and behavioral consequences of TBI. Dr. Rafols is an expert in brain repair and plasticity, having over 35 years of experience in both stroke and TBI models. Dr. Kuhn is an authority on molecular biology and pharmacology of the brain after brain insult. Dr. Mueller is an expert in cardiovascular physiology and will aide in assessing all systemic effects of drug treatments. Dr. Haacke is a leading expert in MRI technique, particularly as it pertains to measurements of blood flow, including CBF. Taken together, the data from these experiments may provide a mechanistic rationale to design a novel therapeutic intervention for TBI aimed at ameliorating the secondary injury resulting from an altered vascular response and hypoperfusion in the brain following brain trauma. Furthermore, as AIM 4 specifically incorporates clinically relevant pharmacological agents, this work is designed to have translation application.

B. BACKGROUND AND SIGNIFICANCE

Traumatic Brain Injury (TBI) and Brain Pathology: Why focus on the vascular response following injury?

TBI results in several major histopathologic events, including among others: cerebral edema which leads to a critical rise in intracranial pressure, diffuse axonal injury which brings about disruption of neural circuits underlying cognitive and motoric behaviors, and alterations in the brain's microcirculation that cause a persistent state of hypoperfusion and improper delivery of vital metabolites to neural tissue. In closed head TBI incidents in humans, all these events are thought to significantly contribute to the ensuing morbidity and mortality encountered in clinical settings. Over 25 clinical trials have been developed aimed at reducing or treating the first two pathologies. None have proven successful in moving forward on a treatment paradigm. However, no one has initiated a clinical trial aimed at alleviating hypoperfusion following TBI. This may, in part, be due to a lack of fundamental knowledge on what causes the altered vascular response following injury. Our laboratory has dedicated the last decade to understanding changes in brain microcirculation following trauma. Using Laser Doppler Flowmetry, albeit a technique with limited scope, our laboratory has shown that TBI results in a significant decrease in blood flow to the brain (Rafols et al., 2007a). This coincides temporally with enhanced vascular stress response (as determined by HSP-70 immunocytochemistry), neuronal injury (Rafols et al., 2007b), and suppressed cellular energy levels in neurons (Huttemann et al., 2007). Furthermore, multiple reports outline the cognitive and psychiatric disorders associated with TBI (Berman et al., 2000; Vakil et al., 2005; Yeats et al., 2005; Ciaramelli et al., 2006). Taken together, this suggests that reduced blood flow following TBI has a profound effect on the general health of neurons, ultimately affecting cognitive outcome. This coupled with our preliminary findings presented in this proposal that show that decreased blood flow leads to an increase in FluoroJade-positively labeled neurons and poor performance on a spatial learning task further emphasizes the potential importance of understanding how decreased CBF following TBI may ultimately contribute to decreased cognition. More importantly, we have also provided preliminary data showing that ameliorating the hypoperfusion following TBI significantly improves the integrity of neurons (i.e., reduced FluoroJade-positivity) and improves behavioral outcome. This further suggests that understanding why and how CBF is altered following TBI may directly lead to effective therapies to treat those suffering the effects of TBI.

Endothelin-1, its receptors and neurons: implications in TBI

The role of the receptors in neurons in different pathological states remains unclear. Several studies have supported a role for ETrA/B in cell death and survival mechanism, respectively. Sato et al (1998) showed that administration of ETrA antagonists resulted in fewer HSP70 labeled cortical neurons after acute cortical neuronal injury. Administration of the ETrA antagonist BQ123 before or after ischemia increased hippocampal CA1 neuronal survival in gerbils subjected to transient global ischemia (Feuerstein et al 1994). While the above reports indicate a role for ETrA in neuronal toxicity, a few reports also support a role for ETrB in neuronal survival. Ehrenreich et al (2000) detected increased apoptosis in neuronal cultures from hippocampus of ETrB deficient rats and increased apoptosis in hippocampal dentate gyrus in association with loss of neuronal ETrB immunoreactivity. Siren et al (2002) found large cortical infarcts and hippocampal apoptosis in ETrB deficient rats subjected to hypoxia-ischemia. While taken together these works support a role for ETrA and ETrB in mechanisms of nerve cell death/survival, it is presently unclear whether the substantial upregulation in neurons after TBI reported here may underlie such mechanisms. In the present model of diffuse TBI, cell death using markers such as activated caspase-3 or TUNEL staining is rarely observed, while cell injury and atrophy as detected by FluoroJade or electron microscopy commonly occur (Rafols et al., 2007b) which would argue against a possible neurotoxic effect by ETrA or enhanced survival by ETrB.

Endothelin-1, its receptors and vasoreactivity: implications in TBI

Since the first submission we now have evidence (see Preliminary Data) that when hypoperfusion is ameliorated in the absence of direct ETrA or B antagonism, neuronal integrity and behavioral outcome significantly improve, suggesting that the observed improvement in neuronal integrity and behavioral outcome following ETrA antagonism is likely due to its effects on CBF and not directly on preventing neuronal cell damage. Therefore, while the possibility of a direct effect of ETrA and ETrB signaling on cell survival cannot be entirely ruled out, more likely control of CBF via these receptors impacts on the metabolic requirements of

neurons, thus influencing the general health of neurons following injury. The regulation of the receptors in neurons seen after TBI, therefore, could be the result of a metabolic demand for their increased synthesis following substantial upregulation of ET-1 and enhanced vasoreactivity of brain reacting microvessels. As such, ETrA/B may be synthesized in neurons and, via the neurovascular unit, transported to the endothelium, thus maximizing ET-1 effects within brain microvessels. This concept is made more plausible by recent findings in our laboratory that ETrA and B are located within astrocytes (Kallakuri et al., in press), the intermediary step between neurons and endothelium (reviewed in Hawkins and Davis, 2005) following TBI.

In peripheral vascular beds, as well as in brain, endothelins are known to exert vasoconstrictive and vasodilatory effects through their actions on ETrA and ETrB (Closel et al 1992, Teerlink et al 1994). Thus, myoplasmic calcium-dependent isometric tension in coronary arteries is mediated by ET-1 via its actions on ETrA (Katwa et al 2000). When ET-1 is released from endothelium it binds to ETrA on vascular SM. Concurrent with ET-1 release, big ET-1, an intermediate peptide is also released and converted to ET-1 by endothelin-converting enzymes (ECEs) on the surface of SM (D'Orleans-Juste et al 1990). The resulting SM contraction is calcium dependent and may be mediated by phospholipase C (Povlishock et al 1983), as well as phosphorylation of SM contractile proteins such as calponin (Kreipke et al 2006, 2007). ET-1 acting through ETrB triggers events similar to those described during activation of ETrA. Thus ETrB activation causes an increase in cytosolic calcium, stimulation of extracellularly-regulated inase pathways (Marsault et al 1990, Lazarini et al 1996) and modulation of cytoskeletal actin organization (Cazaubon et al 1997, Koyama and Baba 1996). However while the vasoconstrictive role of ETrA has been well established, the effect of ETrB activation on microvascular tone remains controversial, with both vasodilatory (Randall et al 1989, Fukuroda et al 1994, Ivy et al 1994, Sato et al 1995) and vasoconstrictive (Clozel et al 1992, Harrison et al 1992, Moreland et al 1992, Teerlink et al 1994) effects being reported.

In spite of these fundamental studies addressing the role of the receptors in vasoreactivity, little is known of the role for the receptors after TBI. Following brain trauma a massive ET-1 increase was detected in periarachnoid cerebrospinal fluid of hypotensive juvenile pigs, with the restoration of the hypotensive response being partially restored by ETrA antagonism with BQ123 (Armstead 1999). Brain parenchymal increases in ET-1 immunoreactivity (IR) and mRNA have been also detected after TBI, these increases being temporally associated with ultrastructural alterations of reacting microvessels and a diminished microcirculation in cerebral cortex (Petrov and Rafols 2002, Rafols et al., 2007). In a previous IR work (Kallakuri et al., 2007) we noted ETrA IR in the peripheral region (i.e, smooth muscle layer) of reacting blood vessel wall from sham-control brains, this IR becoming more intense after TBI. In contrast ETrB seemed restricted to the endothelium in vessels of all sizes, including capillaries. While the contribution of these receptors to cerebral vasoreactivity after TBI is presently unclear, the proposal utilizes both selective and non selective pharmacologic blockade of the receptors after TBI to address these issues.

Endothelin-1 signal transduction- mediated vasoconstriction and Calponin (Cp)

Endothelin-1 (ET-1), a powerful vasoconstrictor, has been shown to be upregulated following injury, this upregulation corresponding to increased vascular contraction after TBI (Armstead, 1996; Petrov et al., 2002; Andresen et al., 2006). ET-1 acts through two G-protein-coupled receptors, A and B (Sakurai et al., 1992). Both result in activation of PKC, however activation of A results in vasoconstriction, while B results in vasodilation (Touzani et al., 1997; reviewed in Jacobs et al. 2006). Armstead (1999) even suggested that ET-1 activation of PKC underlies vascular dysfunction following TBI.

Few investigators have studied the alterations of proteins involved in sustained contractility following TBI. In a broader sense, little work has been done to demonstrate the role of contractile proteins in mediating vasoconstriction in the brain. One such protein known to play a crucial role in vasoconstriction in peripheral vessels is Cp. Cp is an actin-binding protein associated with the contractile machinery in vessels (North et al, 1994). In addition it has been shown to be synthesized in endothelial cells (Birukov et al.,1991; Sakihara et al., 1996; Bandophdhyay et al., 2001). In the relaxed state, Cp inhibits the acto-myosin cross-bridging (Winder and Walsh, 1990a; el-Mezgueldi et al., 1996), thus preventing contraction. In vitro studies have shown that upon phosphorylation, Cp becomes disassociated with acto-myosin, allowing for cross-bridging (Winder and Walsh, 1990b; Tani, 2002). Dephosphorylation of Cp results in a return to the relaxed state of a microvessel (Walsh et al., 1996). In addition to phosphorylation, Cp upregulation seems to increase smooth muscle contraction. Worth et al. (2001) showed that in the contractile state, Cp protein is increased in smooth muscle cells. Two reports showed that application of Cp to cultured smooth muscle cells dose dependently increased contraction (Lin et al., 1993; Yang et al., 2004). Taken together, these data suggest that vasoconstriction

requires both Cp upregulation and phosphorylation and, hence, hypoperfusion following TBI may be the result of both Cp upregulation and phosphorylation.

What is the signal transduction cascade leading to Cp phosphorylation? Calponin contains five potential phosphorylation sites, serine (SER)-175, threonine (THR)-170, 180, 184, and 259 (Kaneko et al., 2000). However, only THR-184 has been shown to significantly affect (increase) vasoconstriction (Nakamura et al., 1993). Naka et al. (1990) showed that Cp is phosphorylated via protein kinase C (PKC). Increasing amounts of PKC applied to cultured smooth muscle cells resulted in vascular contraction (Walsh et al., 1996). Data showing that application of staurosporine, a PKC inhibitor, prevents contraction (Moreland et al., 1992), which provides further evidence that Cp phosphorylation by PKC is critical for vasoconstriction. Therefore, it can be concluded that PKC phosphorylates at the Cp THR184 site, resulting in vasoconstriction (reviewed in Kreipke et al. 2008).

Potential dilemmas in investigating ET-1 effects?

Given that ETrA/B are located in both vascular and neuronal components of brain and that ET-1 potentially exerts effects on both systems, it may be challenging to determine whether antagonists are working via, for example, a direct cell survival/death mechanism or by restoring proper blood flow to brain tissue, thus improving overall neuronal health and ultimately behavioral outcome. However, we will directly test this by ameliorating the hypoperfusion via an ETrA/B independent mechanism. Briefly, we will inject an antibody which blocks Cp phosphorylation at the 184THR site and blocks TBI-induced hypoperfusion. We will then test neuronal integrity and behavioral outcome. Our preliminary data shows that we can ameliorate FluoroJade positive neurons and improve behavioral outcome using this antibody, suggesting that, independent of a direct ETrA/B cell death/survival mechanism, by blocking hypoperfusion we can significantly improve outcome following TBI.

The second dilemma is that, fundamentally one must ask the question how a 40% reduction in CBF could lead to an exacerbation of brain damage following TBI? While the exact mechanism for this is beyond the scope of this proposal and is the source of a follow-up proposal to be submitted for consideration in the next cycle, we hypothesize that initial injury, including diffuse axonal injury (DAI), causes neurons to be compromised (as evidenced by an initial heat shock response which coincides with DAI (Rafols et al., 2007). Then, , the ensuing decrease in critical metabolites and hypoxia that accompanies even relatively modest decreases in blood flow causes further cell injury, as evidenced by our FluoroJade labeling data which is initiated 24 hours after initial insult (Rafols et al., 2007). Therefore it is concluded that initial injury compromises the neuron and secondary injury results, in part, from decreased CBF. This could also explain why a return in normal CBF does not completely block neuronal injury (see Preliminary Data).

Endothelin receptor A antagonists and the clinical setting: promising results for clinical application

Beginning in the early 1990s, endothelin was studied in humans for its potential role in the clinical setting (Vierhapper et al., 1990; Baldys-Waligorska and Szybinski, 1992). Since then, endothelin has been a target for studying a host a pathological states that include disruption of blood flow, including hypertension (Baldys-Waligorska and Szybinski, 1993), hepatorenal syndrome (reviewed in Epstein, 1994), heart failure (Sakai et al., 1996) and decreased cerebral blood flow and hypoxia (Therkelsen et al., 1994). In 1995 Luscher and Wenzel published one of the first reviews which characterized ET-antagonists as potential clinical therapeutics for vascular disorders (Luscher and Wenzel, 1995). In 1999, Benigni and Remuzzi published a follow-up which summarized data from pre-clinical and clinical studies which showed promise for specific ETrA antagonists in controlling hypertension. Bosentan, a mixed antagonist (ETrA and B) was discussed and clinical trial suggested that the potential opposing effects of ETrA and B may render Bosentan less effective (Benigni and Remuzzi, 1999). In 2003, after thorough investigation of ongoing clinical trial, it was reported that, while Bosentan had some success in control of pulmonary arterial hypertension, it was not more effective than other, non-endothelial specific drugs (Krum and Liew, 2003). Once again, this may be attributed to Bosentan being a mixed antagonist (relatively high affinity for both ETrA and B). At the 2007 10th international symposium on endothelin (ET-10) in Bergamo, Italy, several investigators pointed out that while mixed antagonists have had some effects in pre-clinical studies, overall these agents have had little to no effect in the clinical setting. Therefore, it was proposed that specific ETrA antagonists may be more useful.

The first report on a new drug, produced by Actelion Pharmaceuticals, INC in Switzerland, Ro 61-1790 [5-methyl-pyridine-2-sulfonic acid 6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2-1H-tetrazol-5-yl-+ ++pyri din-4-yl)-pyrimidin-4-ylamide] was published in 1997 (Roux et al., 1997). It was found to be 1000-fold more

selective for ETrA than ETrB. It was suggested that Ro 61-1790, which was renamed Clazosentan, may be useful for TBI (Sato and Noble, 1998), ischemia (Dawson et al., 1999), and subarachnoid hemorrhage (Gorlach et al., 2001). In 2006, Clazosentan was included in a clinical trial to prevent vasospasm following hemorrhage (Uhlmann, 2006). Interestingly, this drug has been shown to have little effect in non-brain areas (Vuurmans et al., 2004). Even more promising is Sitaxesentan, with high selectivity towards ETrA (6000X more selective to A than B). This last drug is clinically approved (Battistini et al., 2006). Therefore, selective ETrA antagonism provides a great potential for controlling vascular disruption following TBI. **AIM 4** will directly test whether IV application of ETrA antagonists can improve CBF, neuronal integrity and cognitive outcome after TBI. If successful, this class of drugs may prove effective in the treatment of TBI patients.

C. PRELIMINARY DATA

SPECIFIC AIM 1 tests the hypothesis that TBI causes enhanced ET-1 and endothelin receptors A and B (ETrA, B) expression which causes enhanced vasoconstriction and decreased CBF following TBI.

In figures 1-5 we present preliminary data addressing AIM 1. We have tested the effects of TBI on ET-1 expression and its receptors, A and B. Further, by using antagonists directed at both ETrA and B we have begun to understand the relationship of enhanced ET-1 signaling to CBF following TBI.

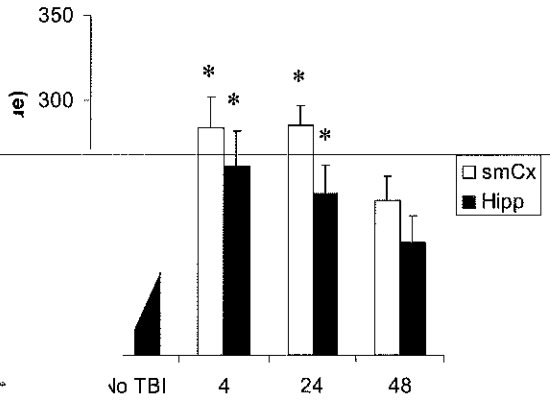


Figure 1. Effects of TBI on ET-1 expression. Concentration of ET-1 following TBI was measured in sensorimotor cortex (smCx) and hippocampus (Hipp) using ELISA. Results indicate that ET-1 is significantly upregulated as early as 4 hours, this upregulation persisting through 24 hours after TBI. N=4 per group, *=p<0.005.

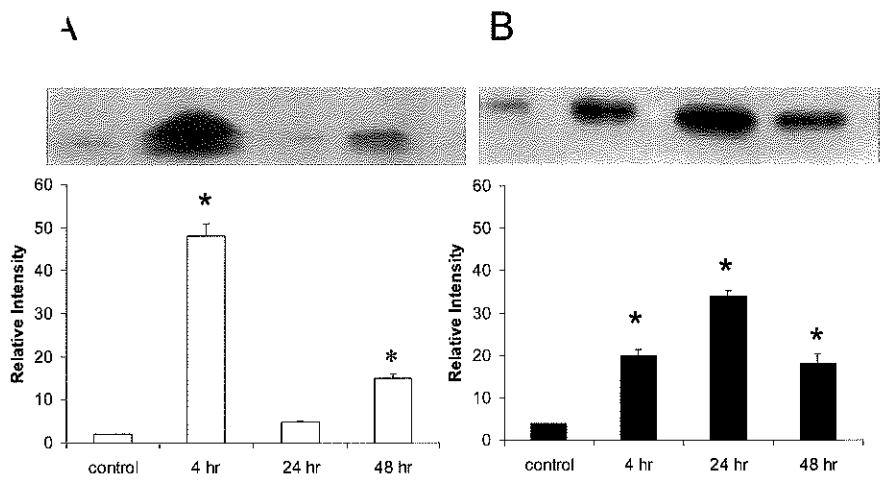


Figure 2. Effect of TBI on ETrA and B expression. ETrA (A) and B (B) expression in smCx vascular tissue (only smooth muscle + endothelium obtained by extraction – see Methods) was determined at 4, 24 and 48 hours post TBI using Western blot analysis. Relative intensities showed that ETrA is significantly upregulated at 4 hours, drops dramatically by 24 hours and rebounds slightly at 48 hours while ETrB is upregulated at 4 hours, this upregulation persisting through 48 hours post TBI.

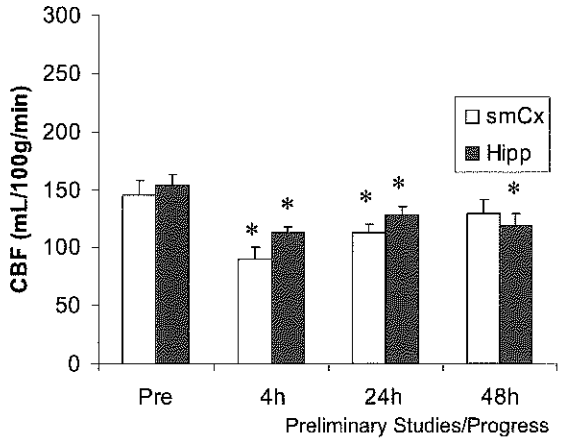


Figure 3. Effect of TBI on CBF. CBF scans using arterial spin labeling functional MRI (ASL-MRI) were taken from 6 animals (Pre TBI). 4 hours later, TBI was induced. 4 hours after TBI (4h) animals were re-scanned for CBF. Scans were repeated at 24 and 48 hours post impact. TBI resulted in a significant (*p<0.05) decrease in CBF which persisted in smCx to 24 hours post TBI and through 48 hours in Hipp.

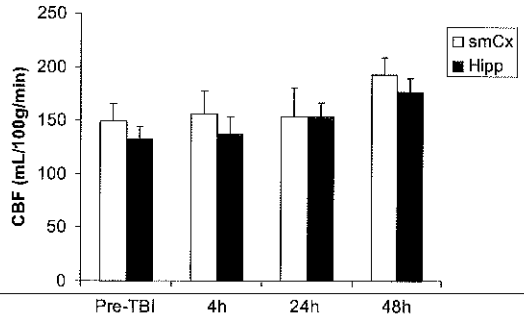


Figure 4. Effect of BQ-123, an ETrA antagonist, on CBF following TBI. CBF measurements were taken using ASL-MRI 1 hour prior to TBI, and at 4, 24, and 48 hours post TBI with IV injection of BQ-123 (0.1 mg/kg) given 30 min post TBI (N=8 per group). BQ-123 was able to ameliorate the hypoperfusion seen following TBI (as indicated by no significant differences in CBF at any of the time intervals observed).

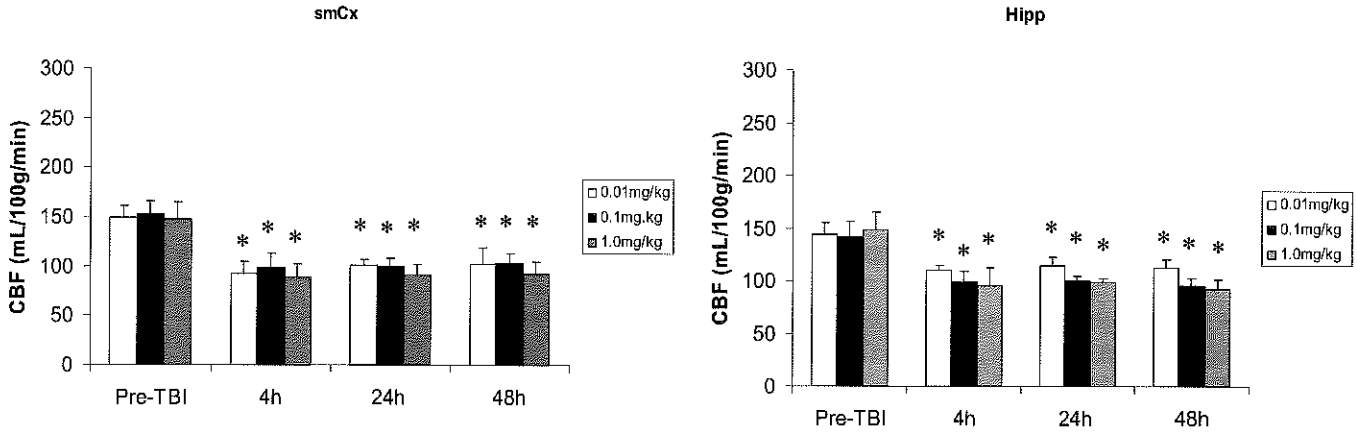


Figure 5. Effect of BQ-788, an ETrB antagonist, on CBF following TBI. CBF measurements were taken using ASL-MRI 1 hour prior to TBI, and then at 4, 24, and 48 hours post TBI with IV injection of various doses of BQ-788 (0.01, 0.1, 1.0mg/kg) given 30 min post TBI (N=6 per group). BQ-788 was NOT able to ameliorate the hypoperfusion seen following TBI (as indicated by a significant difference in CBF at all time points). In addition there was a trend (most pronounced in Hipp) toward a further decrease in CBF with increasing doses of BQ-788. These results suggest that blocking ETrB blocks the vasodilatory ability of microvessels, this inability to vasodilate maintaining the hypoperfusion caused by TBI. *=P<0.005.

SPECIFIC AIM 2 tests the hypothesis that decreased CBF following TBI causes secondary neuronal injury following TBI.

This experiment assesses whether a 40% reduction of CBF following TBI alone is sufficient to cause cell injury or whether TBI is a prerequisite for decreased CBF-induced cell injury . It has been previously suggested that in the presence of DAI and other pathologies associated with TBI, secondary cell injury may occur in the state of "mild" ischemia (reviewed in Siesjo, 1993). Therefore we first tested whether a 40% reduction of CBF without TBI could cause cell injury.

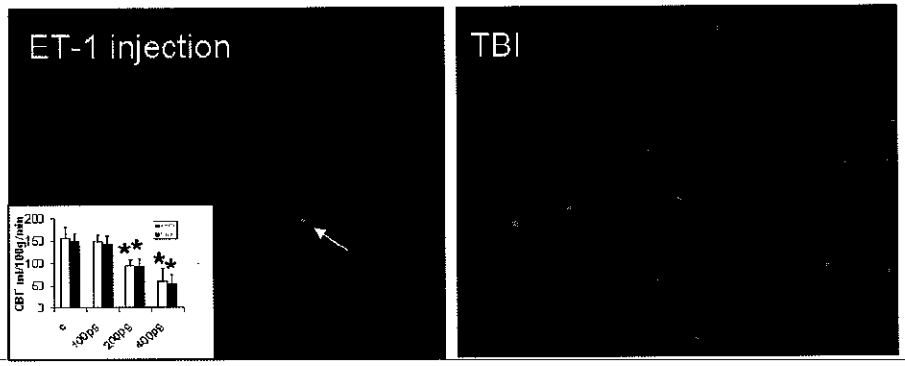


Figure 6. Effect of ~40% reduction of CBF on neuronal integrity both alone and in the presence of TBI. We first determined the appropriate dose of ET-1 that would cause an ~40% reduction in CBF in the non-injured brain. 200pg ET-1 was injected and 4 hours later, coronal sections of brains were obtained for smCx and stained histochemically with FluoroJade (FJ), a marker of cell membrane integrity, to determine the extent of cell injury. TBI brains were dissected at 4 hours post TBI and stained identically. Results show that while ET-1 injection caused negligible FJ staining in smCx, significant FJ staining of cell bodies was found in Layers II-III of smCx after TBI. This suggests that while a 40% reduction in CBF alone is not sufficient to cause cell injury, TBI-induced hypoperfusion causes significant cell injury.

Next, in order to determine whether a TBI-induced reduction in CBF causes neuronal cell damage, we injected the same dose of BQ-123 as in AIM 1 which was sufficient to block hypoperfusion and tested cell integrity using FJ staining. Results (Figure 7) indicate that BQ-123 qualitatively reduces the extent of cell injury after TBI.

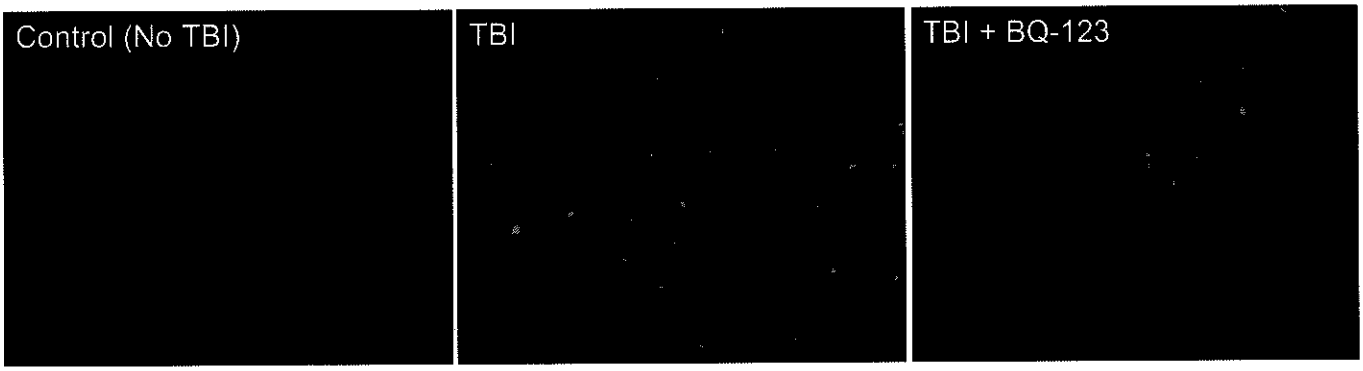


Figure 7. Effect of BQ-123 (shown to block hypoperfusion following TBI), on cell injury following TBI. In order to determine the effect of blocking hypoperfusion on TBI-induced cell injury, we injected 0.1 mg/kg BQ-123 (AIM 1 experiments) 30 min after TBI and measured the extent of cell injury using FJ staining. Compared to TBI, BQ-123 caused a significant reduction in FJ cell labeling in layers II-III of smCx, suggesting that blocking hypoperfusion can improve (albeit not entirely) the amount of cell damage that occurs following TBI. Residual neuronal injury detected in BQ-123 treated animals is likely due to initial insult. Both TBI and TBI+BQ-123 groups were analyzed at 4 hours post TBI.

It could be argued that the effect of ETrA blockade is not due to changes in CBF, but rather to a direct neuronal cell death/survival mechanism (see Background and Significance). In an attempt to block hypoperfusion via a non-ETrA dependent mechanism, we repeated this study using an antibody which blocks the phosphorylation of Cp (at 184THR), thus inducing vasoconstriction. The combined results in Figures 7 and 8, albeit taken from a limited set of animals, indicate that blocking hypoperfusion, whether directly via ETrA antagonism or by directly inhibiting vasoconstriction, reduces the extent of cell injury following TBI.

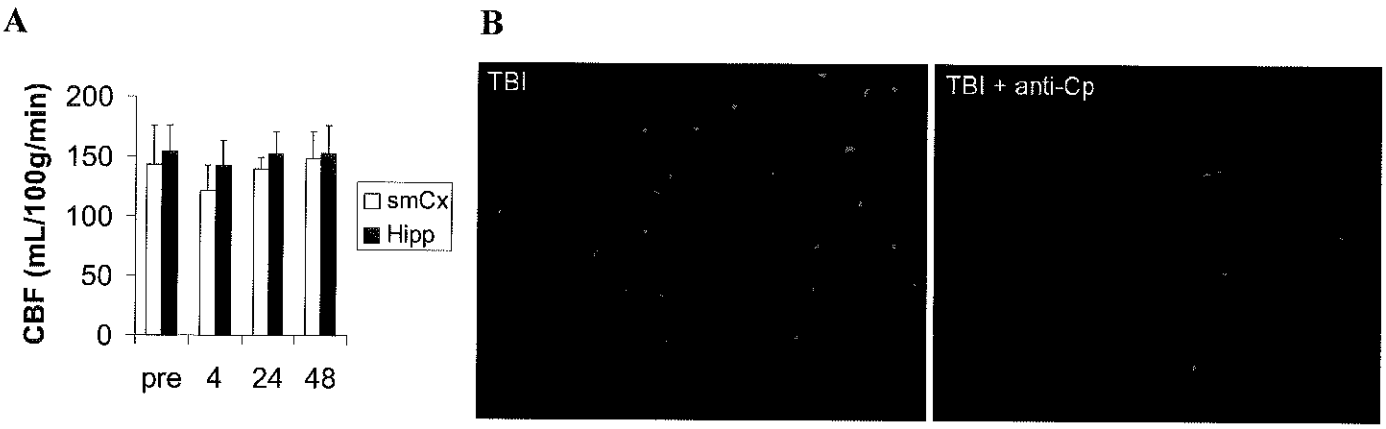


Figure 8. Effect of anti-Cp, which has been shown to block TBI-induced hypoperfusion, on extent of cell injury. *First, CBF measurements were taken in the same regions as used for determination of the effect of the ETrA antagonist (smCx and Hipp) in 3 animals prior to injection or TBI (pre) (A). Next, they were given ICV injection of 20nmol of a peptide generated to bind to Cp at its THR184 phosphorylation site. 1 hour later, TBI was induced. CBF was then re-measured at 4, 24 and 48 hours to test whether the antibody could block hypoperfusion. After it was determined that it could block the hypoperfusion (A), another set of rats received the same injection and then TBI was induced. 4 hours after TBI, animals were sacrificed and tissue sections containing smCx were processed for FluoroJade along side sections from animals receiving only TBI (B). Results indicate that blocking hypoperfusion using anti-Cp sufficiently reduced FJ cell labeling in layers II-III of smCx, and thus, the extent of cell injury.*

SPECIFIC AIM 3 tests the hypothesis that decreased CBF following TBI contributes to cognitive deficits via enhanced neuronal injury.

First we determined the effect of TBI on cognitive outcome.

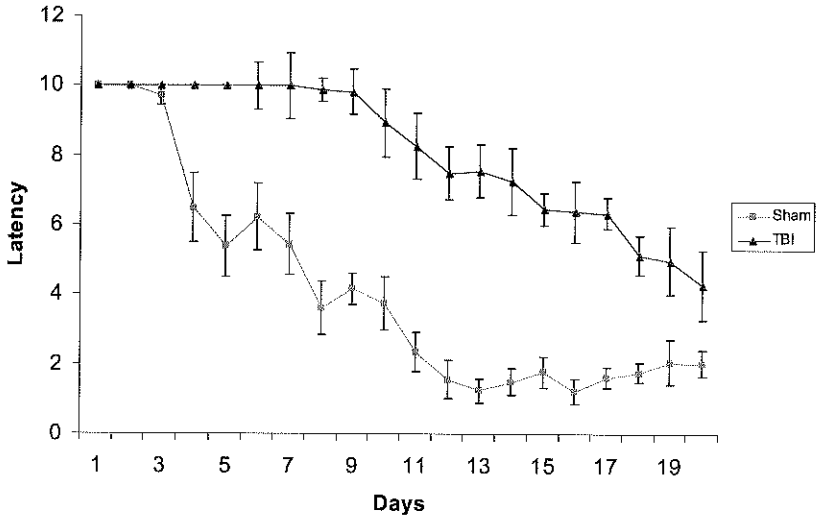


Figure 9 Effect of TBI on cognitive outcome. TBI was induced in 6 animals (TBI group) while 6 were sham operated (NO TBI). 4 hours following TBI, animals were screened for motoric deficits (see General Methods). No animal exhibited such deficits. One day after injury/sham operation, animals were tested for 14 days consecutively for performance on the radial arm maze (for space considerations only latency is shown here). Food intake was also monitored to make sure that lack of motivation to eat was not a factor. All animals ate approximately the same amount. TBI animals showed poorer performance than sham operated animals.

Next we determined the effect of ETrA antagonism on TBI-induced cognitive deficits.

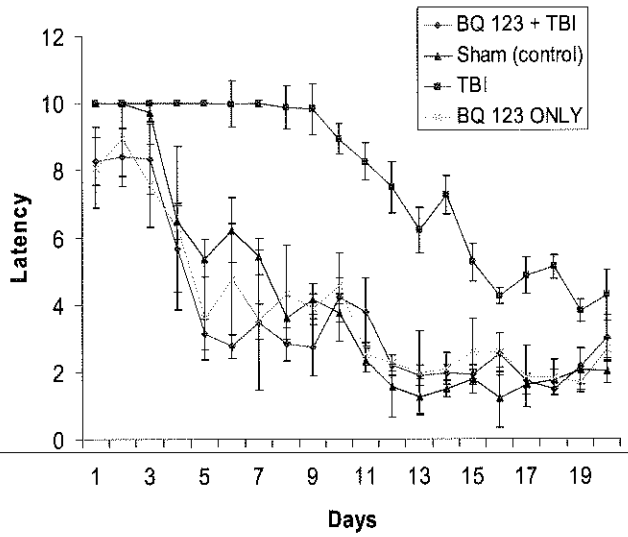


Figure 10. Effect of BQ-123, an ETrA antagonist, on a learning task following TBI. In a set of animals (N=12), TBI was induced. 30 min post injury. 6 animals received no injection while 6 received IV injection of BQ-123 (1.0mg/kg). 6 animals that received sham-operation were used as control. Another 6 were only given BQ-123. All animals were tested for performance on the radial arm maze starting one day following TBI/sham-operation. BQ-123 (ETRA-) was sufficient to improve performance on the radial arm maze compared to TBI animals.

Our results indicate that blocking hypoperfusion via ETrA antagonism improves behavioral outcome after TBI. While we did not see any effect of ETrB antagonism on CBF (Figure 5) we wanted to confirm that a negative response on CBF would correlate with a negative response on cognitive behavior. Figure 11 suggests that blocking ETrB has no effect on behavior.

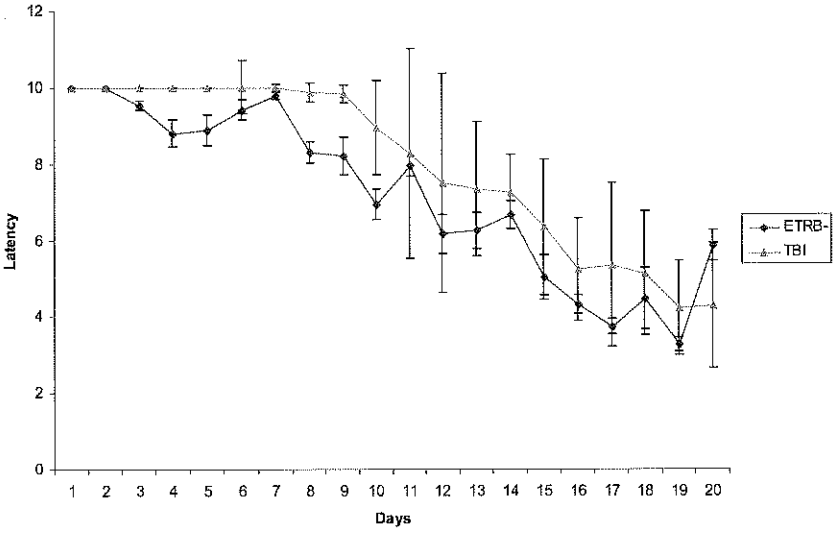


Figure 11. Effect of BQ-788 on cognitive outcome following TBI. 6 animals were subjected to TBI and given IV injection of saline while 6 were given TBI and 0.1mg/kg BQ-788 (which had no effect on CBF) was administered. Behavioral testing commenced. Our results indicate that there is no change in cognitive outcome following TBI when hypoperfusion is allowed to persist.

SPECIFIC AIM 4 tests the hypothesis that IV injection of selective endothelin receptor A antagonists, including Clazosentan, a new drug undergoing Phase II-III clinical trial for ameliorating vasospasm after stroke, and Sitaxesentan, a clinically approved drug, ameliorates TBI-induced decreases in CBF, improves neuronal histopathology and improves behavioral outcome following head trauma.

Here we have determined the effect of IV injection of Clazosentan, a novel ETrA antagonist that has clinical promise, on cognitive behavioral outcome.

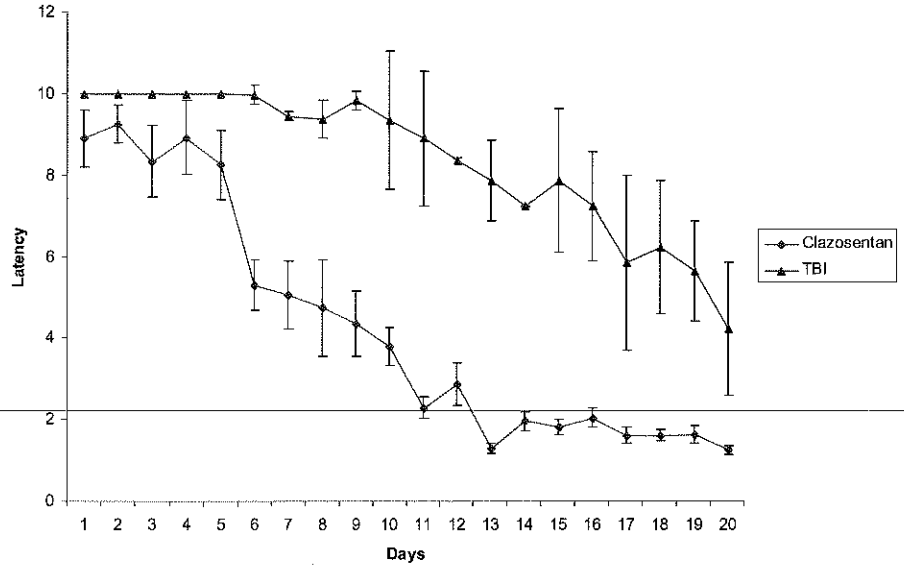
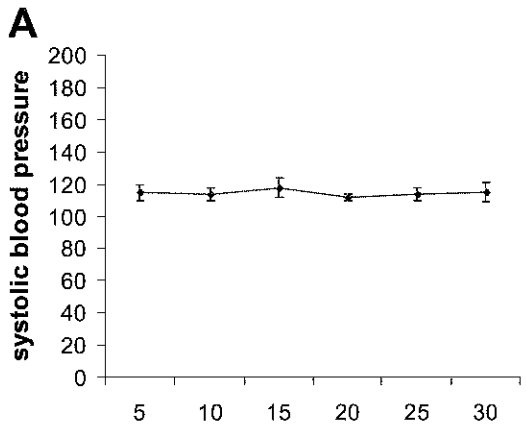


Figure 12. Effect of 1.0 mg/kg IV injection of Clazosentan on spatial learning following TBI. One set of animals (N=5) was subjected to TBI followed 30min later by IV injection of vehicle (TBI) while one (N=7) was subjected to TBI followed 30min later by IV injection of 1.0mg/kg Clazosentan (Clazosentan). One day after TBI, animals were tested in the radial arm maze for latency. Results show that Clazosentan effectively improved spatial learning in the radial arm maze.

Due to the clinical nature of this AIM we have also tested the effect of IV injection of this drug on systolic blood pressure to make sure that there are no peripheral effects of IV administration. Our results indicate that Clazosentan likely has little peripheral effect with respect to blood pressure.



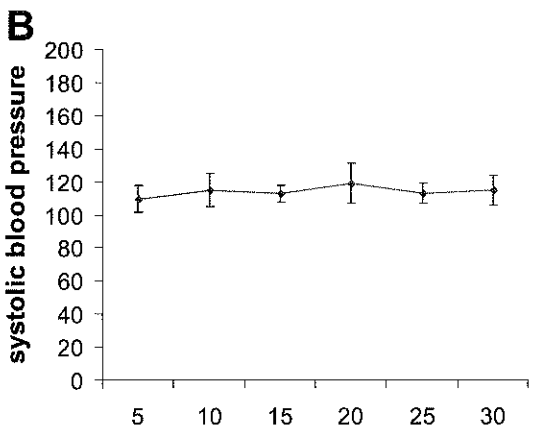


Figure 13. Effect of Clazosentan injection (1.0mg/kg IV) on systemic blood pressure. 3 animals in **A** were given an IV injection of Clazosentan at time point 0 and systolic blood pressure was measured every 5 minutes starting at 5 min and continuing for 30 minutes. No change in blood pressure was detected using a Powerlab™ analyzer. Three animals in **B** were subjected to TBI. 30 min later animals received 1.0mg/kg IV injection of Clazosentan at time point 0. Systolic blood pressure was measured every 5 minutes starting at 5 min and continued for 30 minutes. Animals in B were also given IV injection of 10µg phenylephrine and blood pressure consistently raised to 163 ± 23 (data not shown), suggesting that animals were responsive to vasoactive substances.

In order to more accurately mimic the clinical setting, we have also tried multiple daily injections of Clazosentan given over the duration of hypoperfusion (48 hours). Figure 14 shows the MRI-CBF measurements from 3 animals first prior to TBI/injection and then that were given Clazosentan injections at 30 min post TBI (1st), 24.5 hours post TBI (2nd), and 48.5 (3rd) post TBI. In all cases CBF was measured 30 min following injection.

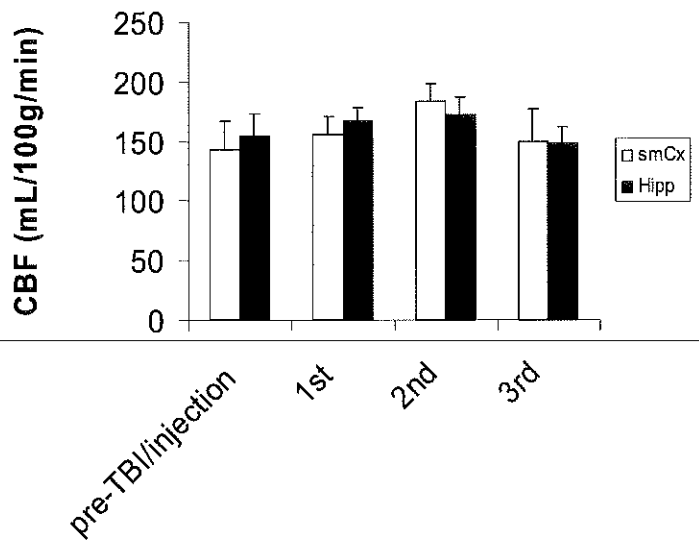


Figure 14. Effect of multiple injections of Clazosentan following TBI on CBF. *CBF was measured in 3 animals. Following this, TBI was induced. 30 min following TBI, animals were given one injection of 1.0mg/kg Clazosentan and 30 min after injection CBF was measured (1st). 24.5 hours post TBI animals were given a second injection of Clazosentan (same dose) and CBF was measured (2nd). 48.5 hours post TBI animals were given another injection of Clazosentan (same dose) and CBF was measured (3rd). Results indicate that there was a slight increase (not significant with N=3) in perfusion rate above control levels upon delivery of the second injection, however this increase was not seen at 48.5 hours, following the third injection. This indicates that control-level CBF can be maintained with multiple injections.*

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

D. RESEARCH DESIGN AND METHODS

SPECIFIC AIM 1 tests the hypothesis that TBI causes enhanced ET-1 and endothelin receptors A and B (ETrA, B) expression which causes enhanced vasoconstriction and decreased CBF following TBI.

Rationale

As discussed in the Background and Significance section, ET-1 has been implicated in the pathophysiology of cerebral vasoreactivity after TBI. While a role for ET-1 signaling in facilitating both direct ETrA/B-mediated cell injury and survival mechanisms has been suggested, our laboratory has focused on its role in mediating vasoconstriction which leads to hypoperfusion of the brain. While AIM 2 will investigate the contribution of hypoperfusion to cell injury, it is essential to establish first cause and effect relationships of ET-1 signaling to overall CBF following TBI. *This entails understanding first the temporal course of the ligand (ET-1) and second, temporal course of the receptors, A and B.* Therefore, in AIM 1 we will first measure ET-1 and and then its receptors levels in discreet areas of the brain (sensorimotor cortex, smCx and hippocampus, Hipp) at various time points following TBI. Next we will determine the extent of vasoconstriction and measure CBF using anterior spin labeling-functional MRI (ASL-MRI) in the same brain regions and at the same time points. Finally, in order to establish cause and effect we will selectively block ET-1 signaling after TBI via either ETrA or B antagonism and re-measure both the extent of vasoconstriction and CBF following TBI. In this way we will determine whether enhanced ET-1 signaling through either ETrA or B causes hypoperfusion following TBI.

Experiment 1.1: Determine ET-1 levels in smCx and Hipp at 4, 24 and 48h post injury.
A series of rats (n=6 per group) will be subjected to TBI and allowed to recover for 4, 24 and 48h post injury. 6 sham-operated animals will be used as control. At the selected time points, animals will be sacrificed and brains harvested, dissected for smCx and Hipp, and frozen at -20°C until processed for ELISA (see General Methods). ET-1 levels will be compared across groups using ANOVA with least significant difference (LSD) posthoc testing to determine whether TBI results in a significant increase in ET-1 and whether this increase is sustained following injury.

Expected Results: Based on observations by others (e.g., Armstead, 1996) and by our provided preliminary data we predict that TBI will result in a significant and sustained elevation in ET-1 levels following TBI.

Possible pitfalls, alternative approaches: Based on the preliminary data we do not predict any pitfalls for this experiment.

Experiment 1.2: Determine ETrA/B mRNA and protein levels in smCx and Hipp vascular and non-vascular (neural) tissue at 4, 24 and 48h post TBI.
In order to determine the temporal course of ETrA/B changes following TBI, using the same tissue in Experiment 1.1, we will process tissue from smCx and Hipp for 1) extraction of smooth muscle and endothelium (SM + EN, see General Methods) and then 2) RT-PCR detection of mRNA and Western to determine protein levels of ETrA/B in vascular and non-vascular brain tissue following TBI (see General Methods).

Expected Results: We anticipate, based on previous findings (Kallakuri et al., 2007) and our provided preliminary data, that ETrA/B mRNA and protein levels will be significantly elevated in both neural and vascular tissue (SM + EN) following TBI.

Possible pitfalls, alternative approaches: While we can distinguish between vascular and non-vascular tissue, if we detect a significant rise in ETrA/B protein in non-vascular tissue we will not be able, using this technique, to appreciate which specific cell types express these receptors. Alternatively, if we see a significant increase in neural tissue, we can utilize double-label immunofluorescence (IF) for receptors and specific cell types (e.g., neurons, astrocytes, oligodendrocytes, etc.). In this way, though it will be difficult to quantify the receptors using IF, we will gain valuable understanding on discreet cellular compartmentalization of these receptors after TBI.

OMB Number. 0925-0001
Expiration Date: 9/30/2007

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. Assurances/Certifications (see instructions)

In agreeing to the assurances/certification section 18 on the SF424 (R&R) form, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the agency's application guide, when applicable. Descriptions of individual assurances/certifications are provided at: http://grants.nih.gov/grants/funding/424

If unable to certify compliance , where applicable, provide an explanation and attach below.

Explanation: Add Attachment Delete Attachment View Attachment

PHS 398 Checklist

OMB Number: 0925-0001
Expiration Date: 9/30/2007

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☐ New ☒ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier: NS064976

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☐

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ☐ No ☐

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March 10, 2009

Christian Kreipke, Ph.D.
Department of Anatomy and Cell Biology
Wayne State University School of Medicine
501 E. Canfield, 9312 Scott Hall
Detroit, MI 48201

I am writing in continued enthusiastic support of your NIH grant proposal entitled "Molecular basis of enhanced contractility following traumatic brain injury: Towards a clinical trial." I truly look forward to my role as Co-Investigator and to continuing our collaboration. Experiments outlined in this grant will provide important new information regarding the mechanisms responsible for deficits in cognitive impairments following traumatic brain injury. As you know traumatic brain injury is a devastating emotional and financial burden to those afflicted and your data offers hope to those individuals suffering from these types of injuries.

I am excited to provide my knowledge and experience to this project. Since laboratories for our group of investigators interested in control of the circulation are located in Scott Hall, the environment is excellent for interactions and cooperative efforts. Your interest and preliminary data on effects of the endothelin antagonist are highly novel and add a new dimension to research efforts in the area of traumatic brain injury. The project outlined in this grant will be very beneficial in establishing you as an independent investigator.

I wish you every success with this interesting and important work. I wish you the best of luck with your final revision of this application.

Sincerely,

Patrick J. Mueller

Patrick J. Mueller, Ph.D.
Assistant Professor of Physiology

WSU MR RESEARCH FACILITY

WAYNE STATE UNIVERSITY

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March 10, 2009

Dr. Christian W. Kreipke
Department of Anatomy and Cell Biology
Wayne State University, School of Medicine
540 E. Canfield, Rm. 9312
Detroit, MI 48201

Dear Chris:

This letter confirms my continued support as a consultant for your NIH application entitled, "Molecular Mechanisms of Enhanced Contractility: Towards a Clinical Trial". It is my pleasure to be part of this proposal and offer my assistance in carrying out the MR elements of this research. You will have access to the WSU 4.7 Tesla animal research scanner in the MRI facility and to the MR technician involved in running the scanner. In addition, I am happy to assist in any MR related data analysis and in preparing manuscripts and grant applications that directly arise from this proposal.

Yours truly,

E. Mark Haacke, PhD

WAYNE STATE UNIVERSITY
SCHOOL OF MEDICINE

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March 10, 2009

Dr. Christian W. Kreipke
Department of Anatomy and Cell Biology
Wayne State University, School of Medicine
540 E. Canfield, Rm. 9312
Detroit, MI 48201

Dear Chris:

I am writing this letter to confirm my continued willingness to serve as a consultant on your NIH application entitled, "Molecular basis of enhanced contractility following traumatic brain injury: Towards a clinical trial". I was encouraged once again by your summary statements and feel that you have answered the critiques and hence have a much stronger application.

I will continue to assist you with characterization of protein changes in your experimental models through the use of immunoblotting/immunostaining. My lab is equipped with a wide array of proteomics instrumentation and we carry out these techniques on a routine basis. We also have considerable experience in determining effective doses in pharmacological studies and I will be happy to assist you in your studies in any way possible.

Our lab is open to you and you we will be happy to provide you with any needed refresher instruction in carrying out the relevant techniques relating to protein expression and modification, and pharmacological determination. Of course, you are also free to use the instrumentation in our lab in coordination with our ongoing studies. I have enjoyed our discussions on your proposal and I am sure that you will uncover many interesting and novel findings. I am looking forward to working with you. Good luck with the review of your final revision of this grant.

Yours truly,

Donald M. Kuhn, PhD

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mechanism on neuronal integrity. Projected at 3 doses X 4 animals = 12 + 4 time points X 6 per time point = 24 = 36
TOTAL: 120
Specific AIM 3

Experiment 3.1: Determine the effect of TBI on cognitive outcome using a spatial learning task. 2 groups X 12 per group = 24

Experiment 3.2: Determine the effect of ICV injection of BQ-123, a selective ETrA antagonist, on spatial learning after TBI. 1 group X 12 = 12

Experiment 3.3: Determine the effect of ICV injection of a peptide which blocks the Cp phosphorylation site (184THR) and blocks TBI-induced hypoperfusion independently of a direct ETrA mechanism on spatial learning after TBI. 1 group X 12 = 12

TOTAL: 48

Specific AIM 4

Experiment 4.1: Determine the effect of IV injection of Clazosentan on CBF, extent of cell injury, and spatial learning following TBI.
CBF: 4 animals
Extent of cell injury: 4 time points X 6 per time point = 24
Spatial learning: 12 animals
Total: 40

Experiment 4.2: Determine the effect of IV injection of Sitaxesentan on CBF, extent of neuronal injury and spatial learning following TBI.
CBF: 4 animals
Extent of cell injury: 4 time points X 6 per time point = 24
Spatial learning: 12 animals
Total: 40

Mean arterial pressure (MAP) studies: projected 16 animals

Multiple dose experiments: projected 72 animals (12 X 6 groups (Clazosentan+veh+veh, Clazosentan+Clazosentan+veh, Clazosentan+Clazosentan+Clazosentan, Sitaxesentan+veh+veh, Sitaxesentan+Sitaxesentan+v, Sitaxesentan+Sitaxesentan+Sitaxesentan))

TOTAL: 80 + projected 88

GRAND TOTAL: 600 (includes animals if multiple-injection paradigm needed) + 10% exclusion (60) = 660

Medical Association (AVMA) Guidelines on Euthanasia. If not, include a scientific justification for not following the recommendations.

Upon termination of a given testing period, rats will be euthanized with a lethal dose of sodium pentobarbital (120 mg/kg IP as above) and death will be assured by bilateral pneumothorax and severing the aorta.

Number of Animals: The number of rats required is based on power studies for the protocols (see Statistics) and on anticipated loss/exclusion of animal subjects due to technical issues such as death upon impact (~5%), motor deficit following injury (~5%). Thus, though not anticipated, approximately 10% more rats may need to be entered into the studies for adequate power to be achieved.

Specific AIM 1

Experiment 1.1: Determine ET-1 levels in smCx and Hipp at 4, 24 and 48h post injury. 4 groups X 6 per group = 24

Experiment 1.2: Determine ETrA/B mRNA and protein levels in smCx and Hipp vascular and non-vascular (neural) tissue at 4, 24 and 48h post TBI. No additional animals needed

Experiment 1.3: Determine the extent of vasoconstriction in smCx and Hipp at 4, 24 and 48h post injury. 4 groups X 6 per group = 24

Experiment 1.4: Measure CBF in smCx and Hipp at -4, 4, 24 and 48h post injury using ASL-MRI. 4 groups X 4 rats = 16

Experiment 1.5: Determine the effect of BQ-123, an ETrA antagonist, on vasoconstriction following TBI. 6 groups (3 doses + veh + TBI alone + no TBI/injection) X 6 per group = 36

Experiment 1.6: Determine the effect of BQ-788, an ETrB antagonist, on vasoconstriction following TBI. 6 groups (3 doses + veh + TBI alone + no TBI/injection) X 6 per group = 36

Experiment 1.7: Determine the effect of BQ-123, an ETrA antagonist, on CBF following TBI. 3 doses + veh X 8 per group = 32

If a multiple-injection approach is needed: 3 groups (BQ-123+veh+veh, BQ-123+BQ-123+veh, BQ-123+BQ-123+BQ-123) X 8 per group = 24

Experiment 1.8: Determine the effect of BQ-788, an ETrB antagonist, on CBF following TBI. 3 doses + veh X 8 per group = 32

TOTAL: 200 ***(224 if multiple injection paradigm is needed)***

Specific AIM 2

Experiment 2.1: Determine the effect of TBI on neuronal integrity. 4 time points X 6 per time point = 24

Experiment 2.2: Determine the effect of a 40% reduction in CBF in the absence of TBI on neuronal integrity. Projected at 3 doses X 4 animals = 12 + 4 time points X 6 per time point = 24 = 36

Experiment 2.3: Determine the effect of BQ-123, a selective ETrA antagonist, on neuronal integrity following TBI. 4 time points X 6 per time point = 24

Experiment 2.4: Determine the effect of ICV injection of a peptide which blocks the Cp phosphorylation site (184THR) and blocks TBI-induced hypoperfusion independently of a direct ETrA

V. VERTEBRATE ANIMALS

The NIH-mandated five points regarding vertebrate animals are addressed as following:

1. Provide a detailed description of the proposed use of the animals for the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex, and numbers of animals to be used in the proposed work.

For all experiments, Male Sprague-Dawley rats (400-450g) will be used. Please see below for break-down of animal number per experiment.

2. Justify the use of animals, the choice of species, and the numbers to be used. If animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and numbers.

The choice to use male Sprague-Dawley rats is based on previous work both in our lab and in the labs cited in the research design. Due to careful use of animals for multiple experiments, no more than 660 male rats will be used in total (see below). Further, rats will be used because of their low cost and because of the large body of information that is now known about their basic neuroanatomy, physiology, and behavior. Rats have an extremely high resistance to infection and are small in size which precludes using large amounts of expensive agents. In addition, the Sprague-Dawley strain has been shown to display pathological changes comparable to those encountered in clinical conditions.

3. Provide information on the veterinary care of the animals involved.

Adherence to IACUC guidelines will be maintained in the experimental treatment and housing of the animals. Housing is provided in an IACUC approved facility in the same buildings as the laboratories (Dr. Kreipke's laboratory and the Department of Animal Laboratory Research Testing Facility). Training in proper care and handling of animals, as provided by the Wayne State University Department of Laboratory Animal Resources, has been successfully completed by the applicant.

4. Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices, where appropriate, to minimize discomfort, distress, pain, and injury.

After brain injury, some animals may experience persisting respiratory difficulties, and will be ventilated as necessary. If this ailment lasts longer than 60 min, such animals will be euthanized with sodium pentobarbital (120 mg/kg, IP injection) consistent with our previous work and with the Panel on Euthanasia of the American Veterinary Medical Association. It is possible that some degree of pain and distress will be present as a consequence of impact on the skull. However, animals are typically awake, but quiet and relatively inactive after trauma. By 1 hour they are usually active and are capable of eating and drinking on their own, although a drop of approximately 7% in body weight is expected. Analgesics will not be used immediately after injury because they (1) interfere with measurements of cerebrovascular function, (2) have neuroprotective effects and (3) in our experience with humans, there is very little or no need for analgesics right after a severe head injury. The effects of analgesics would compromise the results from the proposed experiments.

5. Describe any method of euthanasia to be used and the reason(s) for its selection. State whether this method is consistent with the recommendations of the American Veterinary

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

join her team in Bergamo, Italy in developing a clinical trial testing the effectiveness of using ETrA antagonists such as clazosentan in the treatment of vascular dysfunction following TBI. If successfully funded, this work will provide the pre-clinical data needed to initiate this collaboration, which will quickly translate this work into the clinical realm.

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

mean ± SE. Significance is set at p-value < 0.05. As previously reported (Shen et al., 2007), we were able to detect significant changes in CBF between groups using 6 animals per group with 95% power at α = 0.05.

RT-PCR and Western Analysis. All data pertaining to RT-PCR or Western analysis of mRNA/protein expression are expressed as the average of samples tested independently. Between group analyses are accomplished using one-way analysis of variance (ANOVA) with least significant difference (LSD) post-hoc testing. Data are reported as mean ± SE. Significance is set at p-value < 0.05. As previously reported (abstract in 2006 Society for Neuroscience Annual Meeting, Atlanta, GA; Kreipke 2007c), we were able to detect significant changes in protein expression between groups using 4 animals per group with 95% power at α = 0.05.

Immunofluorescence and immunocytochemistry. All histological and immunocytochemical analyses are conducted using 4 to 6 sections per animal with 3 to 6 areas of analysis per section (see methodology). Data is expressed as an average of each area of analysis. In between group analysis is accomplished using one-way analysis of variance (ANOVA), with least significant difference post-hoc testing. Data are reported as mean ± SE. Significance is set at p-value < 0.05. Based on variability in these data from our previous studies (Kreipke et al., 2006, 2007,a,b) we can distinguish a difference in individual proteins and in capillary density with 95% power at an α level of 0.05 with 4-6 rats per group.

Behavioral Assessments. All behavioral data are expressed as the average latency of completing the task over three trials. Between group analyses are accomplished using one-way analysis of variance (ANOVA) with least significant difference (LSD) post-hoc testing. Data are reported as mean ± SE. Significance is set at p-value < 0.05. Due to the variability in behavior amongst individual animals, we have previously determined (Kreipke 2004, 2007) that we can distinguish significant differences between TBI and control animals using 12 animals per group. Antagonist studies will require 12-15 animals per group to show an improved performance with power of 90%. This allows us to distinguish a difference in latency of 2 min with 90% power at α = 0.05. Additional rats may be required due to failure to exercise, death, or motoric disability following injury.

Project Tentative Schedule:

Table 1. Tentative schedule and task distribution for duration of grant. ***Note that year 5 only represents 6 months so as to accommodate extra time needed in other years for caveats to overcome.***

YEAR	1	2	3	4	5
TASKS	AIM 1-2: determine Et-1, ETrA/B levels after TBI; determine CBF and vasoconstriction after TBI; determine effect of BQ-123/788 on vasoconstriction and CBF after TBI; write up results; present results at National Neurotrauma/Society for Neuroscience	AIM 2: determine effect of TBI, ET-1, BQ-123 and anti-Cp on neuronal integrity; write up results; present results at National Neurotrauma/Society for Neuroscience	AIM 3: determine effect of TBI, BQ-123 and anti-Cp on cognitive behavior; write up results; present results at National Neurotrauma/Society for Neuroscience	AIM 4: determine effect of Clazosentan on CBF, neuronal integrity, and cognitive performance following TBI; determine effect of Sitaxesentan on CBF, neuronal integrity and cognitive outcome following TBI write up results; present results at National Neurotrauma/Society for Neuroscience	AIM 4: determine effect of multiple doses of Clazosentan and Sitaxesentan on CBF and behavioral outcome; present results at National Neurotrauma/Society for Neuroscience; commence writing of clinical trial/further investigation

Future Directions: Clinical trial

As stated in the background and significance section, Benigni and Remuzzi (1999) published one of the first works suggesting that specific ETrA antagonists may provide hope in the clinical setting in ameliorating vascular dysfunction in multiple pathological states. **We have already opened dialogue with Dr. Benigni to**

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compensated, high-resolution, 3D gradient-echo method [20]. Flow compensation ensures that there is no flow-induced phase in SWI-filtered phase images.

Assessment of Cell Injury (FluoroJade histochemistry)
FluoroJade will be performed as previously described (Schmued et al., 1997). Briefly 40 µm sections from smCx and hipp are washed in 80% ethanol with 1% NaOH, 70% ethanol and distilled water. Next tissue sections are washed in 0.06% $KmNO_4$ for 10 min and then washed in distilled water. FluoroJade solution (2 mL) is diluted in 48 mL 0.1% acetic acid and tissue is incubated for 30 min. Section are then rinsed in distilled water, dehydrated, air dried and mounted with a water based mounting media. Sections are analyzed using a Axiotome fluorescence microscope with a Axiocam visualization package.

Assessment of motor deficit:
Since neurological deficits, while rarely seen using this model of TBI, would greatly hinder the ability of a rat to perform on the radial arm maze or in a Morris Water Maze, all animals will be screened following TBI for neurological outcome. In order to screen animals for motor deficit, all TBI animals will be tested using standard neurological function tests, including rotor rod performance, balance beam, and ladder climbing. Based on preliminary screens, rats either performed well or, on the contrary, showed deficit on all tests and, therefore, animals performing at sub-control levels on any test will be grounds for removal from the study.

Behavioral testing and radial arm maze setup:
The rats will be allowed to acclimate to their new environment (in DLAR facility) after their arrival. Then from day 1 to day 3 of the behavioral study the rats will be handled by the researcher for 10 to 15 minutes each. Acclimation to the maze environment also will be initiated during which the rats will be placed on the central platform of the radial arm maze and allowed to roam freely.

A custom designed radial arm maze will be built using black acrylic sheet (0.6 cm thick). Eight identical radial arms are fixed to an octagonal base platform that stands 63 cm above the floor. Each radial arm is 60 cm in length and 10 cm in width with 10 cm – high sidewalls along each arm. At the end of each arm a 5-cm end piece is placed. A hole measuring 2.5 cm in diameter is also cut 5 cm from the end of each radial arm to place a plastic food cup (1 oz).

During behavioral testing, the maze is enclosed within four black linen walls. A white paper triangle (15-cm sides) is placed on one linen wall 10 cm above the base of radial arm #3. An 8" x 11" white paper square with bisecting black lines is placed on the same linen wall 10 cm above the base of radial arm #5. These visual cues are aimed to provide spatial guidance as to the location of the baited arm (i.e. containing the food).

The rats will be tested for the time taken to find the bait (half of a Fruit Loop cereal®) placed in a plastic cup of four different radial arms. Also the number of Type I (entering an unbaited arm) and Type II (re-entering a baited arm after the food has been removed) errors will be recorded. Each rat will be tested daily for three consecutive time trials. The maximum time a rat will be allowed to spend in the maze is ten minutes per trial by the end of which is determined to be conclusion of a trial. Averages of these trials will be calculated and recorded.

Systemic arterial pressure measures
Systemic arterial pressure and HR are measured on a beat-by-beat basis via a Gould P23 XL pressure transducer equipped with an analog-to-digital converter board (Biotech Products) and recorded on computer hard disk for off-line analysis. Data are sampled continuously at 6 Hz by using a DAP 3216a/415 data acquisition processor as the hardware platform.

Statistical Analysis
Note: all statistical analyses will be conducted in accordance with Wayne State University's Biomedical Statistical Core facilities guidelines. Wayne State University's Core facility offers free consultation with a number of their staff at any time during the course of a funded project.

CBF measurements. All data pertaining to CBF are expressed as the average of scans taken independently. CBF is expressed as mL/100g/min. Between group analyses are accomplished using one-way analysis of variance (ANOVA) with least significant difference (LSD) post-hoc testing. Data are reported as

cortical blocks are then postfixed in 1% osmium tetroxide, dehydrated and plastic embedded for ultrathin and semithin (1.0μm) sectioning. Ultrathin sections are stained with uranyl acetate and lead citrate and examined with a JEOL JEM-1010 electron microscope.

One μm thick, plastic embedded sections

1.0 μm thick, toluidine blue-stained plastic sections are obtained from the plastic embedded blocks prior to the ultrathin sections for purposes of block orientation and identification of cortical layers. For quantification of luminal area, twenty five cross-sectioned, EM profiles of microvessels, including capillaries and small arterioles, at a total magnification of X25,000 will be selected from each animal (n=4 animals /group, as above). Profiles of venules are distinguished from those of capillaries on the basis of luminal size, although we acknowledge that it is difficult to identify the transition from a capillary to a vein. Care is taken that the selected profiles are sectioned at nearly perpendicular or at right angles to the long axis of the vessel (i.e., endothelial membrane sectioned mostly perpendicularly to the plane of section). The luminal area (μm^2) is then measured by using the Bioquant IV morphometric system integrated to an IBM-XT computer (R & M Biometrics). Average luminal area/animal group is then calculated by pooling the data from all the microvessels within a group. After data gathering, values between groups are compared using a one-way analysis of variance (ANOVA) test. Where the test revealed significant differences, the least significant difference post-hoc test is used to determine which groups are different. Significance is established at $P<0.05$.

Stereological quantitative analysis of luminal area:

The dissector, an unbiased stereological method (Gundersen, 1988), will be used to determine total luminal area. The dissector consists of two parallel EM sections of know thickness. One of the sections is referred to as the look-up section, whereas the other section is referred to as the reference section. Counting frames are placed over both sections. Any lumen that appears on the reference section, but not the look-up section is counted. The number of counted lumen, Q-, is an unbiased estimate of the number of lumen in the dissector. To determine area fraction (Aa), the counting frame with 121 evenly spaced points (Psect) is placed over both the look-up section and the reference sections and the points that hit the structure (Pstruc) are counted. Aa is derived from the following equation: Aa= Pstruc/Psect = Pstruc/121 and total area = Aa/Q-.

CBF measurements:

Prior to image acquisition, anesthesia will be induced by a steady application of 1% halothane using a specially designed apparatus compatible with the MRI to sedate the animals. The animal will be placed in a prone position on a cradle with a custom-built palate holder equipped with an adjustable nose cone and stereotaxic ear bars in order to minimize movement during MRI scans.

The rat head will be positioned at the isocenter of a magnet. MRI scans will be repeated at four time points. Baseline scans will be run before TBI is induced, and then at the 4th hour, 24th hour and 48th hour post-TBI. All MRI measurements will be performed on a 4.7-T horizontal-bore magnetic resonance spectrometer (Bruker AVANCE) with an 11.6-cm-bore actively shielded gradient coil set capable of producing a magnetic field gradient of up to 250 mT/m. A whole-body birdcage radiofrequency (RF) coil (inner diameter, 72 mm) will be used as the transmitter for homogeneous RF excitation, and a surface coil (30 mm diameter) will be used as the receiver, with active RF decoupling to avoid signal interference. Four sequences will be run in this set of experiments: T2-weighted imaging, T1-weighted imaging, and ASL for the measurement of flow and SWI to measure changes in oxygen saturation and flow, as well as for the determination of evidence of vascular damage and hemorrhage.

For all sequences, the field of view will be 40X40X24 mm^3; thus, the whole brain will be imaged. The remaining imaging parameters used are as follows:T2-weighted imaging Rapid Acquisition with Relaxation Enhancement (RARE): TR=4751 ms, TE=46 ms, matrix size NxXNy =256X256, number of slices (Ns)=24 (thickness, 1 mm), Nacq=1; T1-weighted imaging (3D Fast Low Angle Shot [FLASH]): TR=22 ms, TE=7 ms, flip angle (FA)=58 and 208, matrix size NxXNyXNz =256X256X24, Nacq=1(FAs 58 and 208 will be used to calculate T1 maps); ASL: TR = 1550 ms, TE = 7.65 ms, matrix size NxXNy =128X70 (interpolated by zero filling in k-space to 256_256), slice=1 (thickness, 2 mm), Nacq=2, labeling slice=2 cm offset from isocenter, adiabatic fast passage with Magnetization transfer contrast (MTC) gradients=1.5 s, spin echo=3; SWI: TR=36 ms, TE=15 ms, FA=208, matrix size NxXNyXNz =512X512X24, Nacq=2. SWI is based on a fully flow-

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throughout the brain we do not foresee problems in interpreting the data. That is, the extent of effect will be known based on MRI scans.

Extraction of smooth muscle/endothelium from whole brain preparations:
In all cases, gene and protein levels will be measured in the areas specified in each AIM in tissue containing neurons, neuropil, vascularization, etc. and this will be compared with data taken from just the vascularization. In order to assess gene and protein levels in vascularization we will first remove brain and cut 2-mm thick slices containing the region of interest. Next we gently remove meninges, collect cortex and subcortex cut tissue into 1 mm segments and homogenize tissue in 3 ml ice-cold PBS using a Dounce homogenizer (30 strokes for loose grinder and 25 strokes for tight grinder). Then we spin for 3500 g for 10 min at 4 ^{0}C, then resuspend in 3 ml PBS. Next we filter the tissue suspension through a 41-µm nylon mesh (Spectrum), wash the mesh three times with 5 ml PBS. We usually use a small vacuum pressure to help the filtration). Microvessels are retained on the mesh, and so we wash them off with PBS, spin 3000 g for 10 min at 4 ^{0}C to pellet the microvessels and re-suspend the pellet in 15% dextran T-500, applying the suspension onto 20% dextran T-500. Finally, we spin at 25,000g for 10 min, collect the pellet as microvessels.

Real-time PCR:
Real-time PCR following a previously optimized protocol (Guo et al., 2007) will be used to determine gene expression. Briefly, total RNA is isolated by using STAT-60 Reagent (Tel-Test Co., TX, USA) according to the manufacturer's protocol. Next, the samples are purified using the RNeasy kit (RNeasy Mini Kit, Qiagen, MD, USA) and DNase Treatment & Removal Kit (Ambion, CA, USA). Random primers are used to create first-strand DNA synthesis using iScript cDNA synthesis kit (Bio-Rad). The cDNA is then amplified using an ABI Prism 7900HT sequencing detection system for real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). To determine the relative quantification of target gene expression, we used the comparative CT (threshold cycle) method with arithmetic formulae (Heid et al., 1996, Gibson et al., 1996, Hiratsuka et al., 1999; Ding et al., 2003).

ELISA detection of ET-1:
ET-1 expression will be determined as previously published (Petrov et al., 2002) using ELISA. Briefly, tissue containing smCx or Hipp is extracted. Amount of ET-1 will be determined using a colorimetric method after detection of the protein with a kit purchased from R&D systems (Minneapolis, MN, USA).

Western analysis:
Whole brains will be harvested (n=4 animals per group), placed in cold methylbutane on dry ice and partially frozen. Brains will then be dissected to isolate smCx and hipp. Isolated smCx and hipp will be homogenized in Lamelli's solution and subjected to SDS-PAGE. Protein concentrations will be standardized for all samples. Electrophoresis will run at 40mA for the first 40 min and then 20mA for 3 hrs. Gels are transferred to nitrocellulose paper, and blocked using 1% nonfat dried milk in TTBS at room temperature for one hour. Transferred samples will then be incubated in primary antibody (individual antibodies listed as needed per experiment) in TTBS at 4^{0}C overnight and then incubated in secondary antibody and donkey serum in TTBS at room temperature for 30 min. Nitrocellulose will be rinsed in Lumiglow solution for 90 seconds, exposed to X ray film and developed. Band intensity of immunoblots will be quantified using optical densitometric (OD) analysis.

Electron Microscopy (EM):
Vasoreactivity will be determined using EM and measurement of luminal area as previously described (Rafols et al., 2007). Briefly sham-operated controls and experimental (post ET-1 injection at various time intervals) animal groups (n=4 animals/group) will be anesthetized with 2.5% halothane. Animals will be perfused through the ascending aorta with isotonic saline (50 cc) followed by a 200 cc solution containing 4% paraformaldehyde and 0.1% gluteraldehyde in 0.1 M phosphate-buffered saline (PBS). Following perfusion, each brain is carefully resected from the skull and stored in the same perfusion fluid at 4^{0}C overnight. Each brain is then sectioned coronally at 300 µm with a vibratome. Sections containing the sensorimotor cortex (-1.5 to -3.5 mm from bregma, Paxinos and Watson,1998) are further trimmed by two consecutive vertical cuts from the pial surface to the subcortical white matter, and a longitudinal cut immediately below the cortical gray matter. Isolated

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Experiment 4.2: Determine the effect of IV injection of Sitaxesentan on CBF, extent of neuronal injury and spatial learning following TBI. This experiment will be conducted as in Experiment 4.1, however Sitaxesentan will be used in lieu of Clazosentan. Dose response will remain the same.

Expected Results: Since Sitaxesentan has the highest affinity of the "-sentans" to ETrA, and based on the fact that it has passed clinical trial (September 2007) and is clinically available, we predict that this drug will have the best success in ameliorating hypoperfusion following TBI, and improving both cell integrity and behavioral outcome.

Possible pitfalls, alternative approaches: As in experiment 4.1, while Sitaxesentan is a clinically approved drug, no one has tested its usefulness after TBI. Therefore we may have to adjust the dose response curve or perform multiple injections as in experiment 4.1.

NOTE: *Since drugs in this AIM will be delivered IV and since we are developing data to move towards clinical trial, in addition to the aforementioned measures, an additional set of animals (N=4 per group) will be used for each experiment in which systolic blood pressure and heart rate will be monitored either through catheter placement or via telemetry (see General Methods). Since none of these antagonists have been shown to have significant adverse effects in normal animals or in ongoing human clinical trials, we do not predict that following TBI there will be a problem. However, we will test this nonetheless to shed more light onto any possible side effects of these drugs.*

GENERAL METHODS

Closed head trauma model:
Adult male Sprague-Dawley rats (400-450) (Sprague-Dawley) will be anesthetized with 5% halothane in 2% oxygen prior to intubation, and then maintained on 1.5% halothane via a mask and spontaneous breathing. Halothane will be used as the anesthetic for all experiments. The use of halothane instead of the more recently introduced isofluorane is preferred because of recent evidence indicating the latter neuroprotective properties (published data [Zhao et al., 2007; Wei et al., 2007] and more recent data presented at the Brain '07, International Cerebral Blood Flow and Metabolism meeting held in Osaka, Japan). A midsagittal scalp incision will be performed and the underlying muscles retracted laterally. Cranioplastic cement will be used to attach a 10mm diameter X 3 mm thick, round metal helmet directly to the skill over the sagittal suture and between the coronal and lambdoidal sutures. The helmet is used to distribute the applied force over the surface of the parietal bones, thus preventing skull fractures with penetrating brain injury. After the cement is allowed to dry for three minutes, the animals will be placed prone on a platform as described in the acceleration impact trauma model of Marmarou (Marmarou et al., 1994). After 30-40 seconds of placement, 450g of weight contained in a hollow plastic cylinder will be dropped directly onto the helmet from a height of 2 meters. Following a brief convulsion and respiratory arrest, most animals restart breathing on their own. However, in some cases, the use of a rodent respirator or CPR is necessary prior to spontaneous breathing. Using these precautions, in our hands mortality has been reduced to less than 5%. In some animals after impact, the helmet will be removed and the skin sutured only if the skull shows no evidence of fractures. After suturing the skin, sensory cutaneous and evoked motor responses will be tested. Usually the intubation tube is removed at 10 minutes post trauma and only animals which are able to right themselves before 30 minutes after injury will be included in the study (Petrov et al., 2000; Petrov et al., 2002a). Brain and leg muscle temperatures will be taken routinely, in some instances up to 24 hrs post injury. We have determined that brain temperature fluctuated only 1.5°C, and muscle temperature 1.3°C, during this time period.

ICV versus IV injections:
Every attempt has been made to use IV injections for antagonist delivery in order to mimic more closely the clinical setting. However, due to the known effects of both ET-1 and Cp in peripheral (i.e., non-brain) vascular beds, we will use ICV injections of ET-1 and anti-Cp to test mechanisms related to hypoperfusion. While the possibility of differential effects exists due to differences in diffusion between IV and ICV injections, since efficacy of both ET-1 and anti-Cp is based on CBF and since we can measure CBF in discreet areas

peptide, induce TBI, and measure outcome using the radial arm maze as before to determine whether blocking TBI-induced hypoperfusion independent of a direct ETrA mechanism can improve cognitive outcome.

Expected Results: We predict that blocking Cp-mediated vasoconstriction will improve spatial learning on the radial arm maze.

Possible pitfalls, alternative approaches: Same as in experiment 3.1.

SPECIFIC AIM 4 tests the hypothesis that IV injection of selective endothelin-A receptor antagonists, including Clazosentan, a new drug undergoing Phase II-III clinical trial for ameliorating vasospasm after stroke, and Sitaxesentan, a clinically approved drug, ameliorates TBI-induced decreases in CBF, improves neuronal outcome and improves behavioral outcome following head trauma.

Rationale

The idea of using ETrA antagonists is becoming more and more a reality in the clinical setting (Benigni et al., 2007). At the recent ET-10 conference held in Bergamo, Italy, several leading authorities advocated their use in controlling hypertension, relieving the deleterious effects of stroke, and controlling vasospasm (Benigni et al., 2007). However, to date no one has explored the clinical application of selective ETrA antagonists following TBI. We have been in dialogue with the companies (Actelion and Pfizer) involved in the production of both Clazosentan and Sitaxesentan, respectively which are eager to determine whether these drugs can elicit positive effects after TBI. Therefore, this AIM is designed to help provide a sound rationale for and to stimulate the use of ETrA antagonists as a therapeutic intervention to help ameliorate the deleterious effects of TBI. Each experiment will test different ETrA antagonists which were chosen based on their selectivity to the ETrA receptor over the ETrB. While ETrA is associated with vasoconstriction, ETrB is associated with vasodilation. It has, thus, been suggested that "mixed" antagonists have limited clinical value. This, combined with our provided data that ETrB antagonists do not improve CBF or cognitive outcome dampened our original intent to try Bosentan, a mixed ETrA/B antagonist. However, Clazosentan is 1000X more selective to ETrA and Sitaxesentan is 6500X more selective to ETrA (Battistini et al., 2006). By not only testing its effects on CBF but also neuronal integrity and spatial learning, we hope to provide sound mechanistic rationale and evidence for the effectiveness of ETrA antagonists that clinical trials can commence to help treat TBI.

Experiment 4.1: Determine the effect of IV injection of Clazosentan on CBF, extent of cell injury, and spatial learning following TBI.

Expected Results: Since Clazosentan has a high affinity towards ETrA and based on our preliminary data and the fact that this drug has been successful in Phase II clinical trial for ameliorating vasospasm after stroke, we predict that Clazosentan will be successful in ameliorating hypoperfusion after TBI, and improving both neuronal integrity and spatial learning on the radial arm maze.

Possible pitfalls, alternative approaches: Since no one has used this drug in TBI models, we may have to adjust the dose response curve to optimize the efficacy of the drug. *Furthermore, though our preliminary data suggests that a single dose of Clazosentan is effective in ameliorating decreased CBF, to more accurately mimic a potential clinical setting we will be prepared to offer daily injections during the time of hypoperfusion (up to 48 hours for a total of 3 injections). In this experiment we will test the following groups: Clazosentan+veh+veh, Clazosentan+Clazosentan+veh, Clazosentan+Clazosentan+Clazosentan. Injection will be given at 30 min,, 24.5 hrs and 48 hrs post TBI.* In order to minimize animals used and most efficiently utilize our time, we will use CBF as our measure of efficacy. If multiple injections are needed we can measure CBF daily using MRI, thus testing the efficacy over time. As we have already shown, ASL-MRI measures can be easily taken with little harm to the animal and, thus, when a dose is achieved which blocks hypoperfusion after TBI, we will use that dose to determine its effects on cognitive outcome. Furthermore since our preliminary data suggests that a 0.1mg/kg IV dose of Clazosentan can ameliorate the known hypoperfusion following TBI, we do not expect difficulties in producing positive results in this experiment.

Rationale
In several of our published works we have suggested that decreased CBF following TBI leads to cognitive deficits (see Rafols, 2007 for review). However, we have been criticized by several groups because of the lack of cause-effect relationship between decreased CBF and poor cognitive outcome. Many TBI investigators, for instance, feel that TBI causes a decrease in metabolic demand which in turn causes a decrease in CBF. While this is likely the case, what has yet to be explored is how the decrease in CBF could, in turn, lead to long lasting cognitive deficits. If, for instance, TBI causes initial cell injury (as evidenced by our work and by work of others [see Rafols, 2007 for review]), but then decreased CBF causes secondary cell injury (as evidenced in preliminary data pertaining to AIM 2), then the hypoperfusion could indirectly cause cognitive deficits through enhanced cell injury. Therefore, we hypothesize that by reducing the extent of hypoperfusion, neuronal integrity would be improved (AIM 2), leading to improved cognitive outcome following TBI, the focus of this AIM. Hence, in AIM 3 we will first determine the effect of TBI on cognitive outcome using a radial arm maze test of spatial learning. Next, as in AIM 2, we will test the effect of blocking hypoperfusion following TBI, either by using ETrA antagonism or a peptide designed to block Cp-mediated vasoconstriction, on performance in the same radial arm maze task. Data across groups will be compared using ANOVA with LSD posthoc testing to determine whether decreased CBF contributes to overall cognitive deficits following TBI.

Experiment 3.1: Determine the effect of TBI on cognitive outcome using a spatial learning task. TBI will be induced in 12 animals. Sham operated (N=12) animals will serve as control. Following a one-hour recovery period, animals will be screened for neurological deficits (see General Methods). On the following day, animals will be tested on a radial arm maze for latency of retrieving food, type I (entering an unbaited arm) and type II (re-entering an arm in which food was previously taken) errors (see General Methods for further clarification). Testing will continue for 20 days following TBI/sham operation. Performance of TBI versus sham operated animals on the maze, which takes into account latency and errors, will be compared to determine the effect of TBI on cognitive outcome.

Expected Results: Based on our preliminary data we expect that TBI will significantly impair cognitive outcome.

Possible pitfalls, alternative approaches: A caveat here is that the radial arm maze is just one assessment of spatial learning and cognitive outcome. Are other spatial learning tasks similarly compromised after TBI? In order to address this issue and to confirm our results with the radial arm maze, we are prepared to repeat this study using the Morris water maze, which is another widely accepted cognitive test in rodents available to us in the Anatomy Department.

Experiment 3.2: Determine the effect of ICV injection of BQ-123, a selective ETrA antagonist, on spatial learning after TBI.
Animals (N=12) will be treated as in Experiment 3.1, however 30min following TBI BQ-123 will be administered (effective dose based on CBF measurements is determined in AIM 1). Behavioral testing will commence as in Experiment 3.1 and results will be compared to those obtained in 3.1 using ANOVA with LSD posthoc across 20 days of testing to determine whether BQ-123 if effective in improving spatial learning following TBI.

Expected Results: Based on preliminary findings, we expect that BQ-123 will improve behavioral outcome as compared to vehicle injected TBI animals.

Possible pitfalls, alternative approaches: Same as experiment 3.1.

Experiment 3.3: Determine the effect of ICV injection of a peptide which blocks the Cp phosphorylation site (184THR) and blocks TBI-induced hypoperfusion independently of a direct ETrA mechanism on spatial learning after TBI.
As mentioned in AIM 2, it is possible that positive results in Experiment 3.2 may be independent of CBF (i.e., ETrA has been linked to cell death/survival mechanisms). While this would not diminish the goal of testing whether ETrA antagonism can improve behavioral outcome following TBI, it would diminish enthusiasm for the overall hypothesized mechanism which involves CBF. Therefore, here we will (N=12 rats) administer the Cp

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survival mechanisms and, therefore, any direct effects may not be detected. Further, our provided preliminary data does not show appreciable cell injury with ET-1 injections, alone. In any case, we would be prepared to select another vasoconstrictor, such as vasopressin, and repeat the experiment to confirm that any changes that may be detected are likely due to CBF changes and not to ET-1 elicited changes in neurons.

Experiment 2.3: Determine the effect of BQ-123, a selective ETrA antagonist, on neuronal integrity following TBI.
We will inject BQ-123 (effective dose determined in AIM 1) 30min after TBI to block hypoperfusion. Brain tissue will be collected and stained for FluoroJade (FJ) to determine the extent of neuronal injury. This data will be compared with that in Experiment 2.1 to test the effect of blocking TBI-induced hypoperfusion on neuronal integrity following injury.

Expected Results: Based on preliminary results, we predict that BQ-123 will decrease the extent of neuronal injury seen following TBI.

Possible pitfalls, alternative approaches: As already mentioned, it is possible that BQ-123 will block a direct ETrA cell death mechanism. However, this is unlikely as we have not detected cell death in this model of TBI. Nonetheless, we have introduced the next experiment to block hypoperfusion following TBI independently of ETrA to rule out a direct ETrA cell death/survival mechanism.

Experiment 2.4: Determine the effect of ICV injection of a peptide which blocks the Cp phosphorylation site (184THR) and blocks TBI-induced hypoperfusion independently of a direct ETrA mechanism on neuronal integrity.
While Cp contains several potential phosphorylation sites, Nakamura et al. (1993) demonstrated that only THR184 phosphorylation was critical for eliciting vasoconstriction. Therefore, we had a peptide created by Invitrogen, Inc. to block the following sequence which contains the THR184 sequence: 177FASQQGMTA185. We will inject this peptide into the ventricles (ICV; 100ng) 1 hour prior to TBI to block hypoperfusion independently of a direct ETrA mechanism and measure CBF as before. This data will be compared to that in experiment 1.4 to confirm that we sufficiently blocked TBI-induced hypoperfusion. Brain tissue will be collected and stained for FluoroJade to determine the extent of neuronal injury. This data will be compared with that in Experiment 2.1 to test the effect of blocking TBI-induced hypoperfusion independent of a direct ETrA mechanism on neuronal integrity following injury.

Expected Results: Based on preliminary results, we predict that when delivered at a concentration sufficient to block hypoperfusion following TBI, the anti-Cp peptide will decrease the extent of neuronal injury seen following TBI.

Possible pitfalls, alternative approaches: We acknowledge that this approach has only been conducted in our laboratory (Kreipke et al., 2008) and hence, while novel, may need refinement. A potential problem, for instance, is that ICV injection may not yield the same diffusion in the brain as IV injection (as used for ETrA). However, as discussed in General Methods, the choice to use ICV injections is based on the fact that Cp-mediated vasoconstriction is critical for normal heart rate (see Kreipke et al., 2008 for review) and, hence, blocking this will likely compromise animals. Secondly, we may have to adjust the dose of the peptide, for example, given in order to see an effect. *Furthermore, we may need to give multiple injections in order to maintain blood flow.* Efficacy and duration of peptide administration will be tested based on CBF measurements since, as previously described, these measurements are quickly and reliably achieved. However, as blood flow can quickly and repeatedly be measured using ASL, we do not predict any problems in determining a dose of anti-Cp which sufficiently blocks hypoperfusion. Since we have preliminary data which strongly suggests that anti-Cp does block TBI-induced hypoperfusion in both smCx and Hipp and cell injury, we do not predict any major problems with this experiment.

SPECIFIC AIM 3 tests the hypothesis that decreased CBF following TBI contributes to cognitive deficits via enhanced neuronal injury.

Our previously published observation using Laser Doppler Flowmetry (LDF) to measure superficial blood vessels in smCx (Rafols et al., 2007) combined with our more recent data using ASL-MRI show that TBI causes approximately a 40% reduction in CBF, this hypoperfusion being sustained through 48h post injury. However, this reduction is CBF may not be typically thought of as sufficient to cause cell injury (reviewed in Ding et al., 2006). However, in the presence of diffuse axonal injury and other pathologies associated with TBI, we posit that it is possible that a 40% reduction of CBF following initial insult may further compromise neurons. Therefore, the overall goal of the AIM is to determine whether the amount TBI-induced hypoperfusion in our model is sufficient to cause cell injury. In order to test the hypothesis that decreased CBF following TBI can cause secondary neuronal injury, we will first determine the extent of cell injury following TBI. We have chosen the histochemical stain FluoroJade (FJ) as our marker of cell *injury which may not necessarily lead to cell death.* Previously we published data on cell death using TUNEL and activated caspase-3 immunoreactivity and determined that while there is little to no cell *death* in our model of TBI, abundant nerve cell injury was detected by both FJ staining and EM (Rafols et al., 2007b). Next, we will determine by using microinjections of ET-1 at a level which we will determine using ASL-MRI causes approximately the same reduction in CBF (~40%) in the absence of TBI. We will measure the extent of cell injury using FJ cell labeling and compare these results to determine whether a 40% reduction is sufficient to cause cell injury or whether TBI is a requisite for such damage. After we have established this relationship, we will then block hypoperfusion after TBI using the same ETrA antagonist as in AIM1. FJ labeling will be conducted and results compared to those obtained for TBI only to determine whether TBI-induced hypoperfusion causes cell injury. As discussed in the Background and Significance section, a potential confound to this experiment is that ETrA has been suggested to directly participate in cell death mechanisms which, by blocking ETrA we could be improving cell injury independently of changes in CBF. Therefore, in a final experiment in this AIM we will use a novel approach to blocking hypoperfusion independent of ETrA by administering an antibody which blocks the 184THR site of Cp, which is critical for vasoconstriction. In doing so, we can determine whether decreased CBF as a result of TBI causes cell injury.

Experiment 2.1: Determine the effect of TBI on neuronal integrity.
6 animals per group will be subjected to TBI and smCx and Hipp will be collected at 4, 24 and 48h post injury. 6 animals will undergo sham operation for control. Serial coronal sections throughout the smCx and Hipp will be collected and stained with FJ. FJ-positive neurons will be manually counted and averaged per animal to determine the extent of cell injury following TBI. Averages will be compared across all groups (sham + all time points) using ANOVA with LSD posthoc to determine whether TBI causes cell injury.

Expected Results: Based on the provided preliminary data we expect to see considerable cell injury following TBI.

Possible pitfalls, alternative approaches: We acknowledge that proper stereology will be required for this experiment in order to accurately quantify FJ-positivity. However, as Drs. Kreipke, Rafols, and Kuhn all have extensive experience in quantifying cell injury we do not foresee any problems.

Experiment 2.2: Determine the effect of a 40% reduction in CBF in the absence of TBI on neuronal integrity.
First we will determine a dose of ICV injection of ET-1 which causes an approximately 40% reduction in CBF in normal (No TBI) animals. CBF will be measured as in AIM1 using ASL-MRI. Once this dose is determined, we will inject the drug, wait 4 hours and then collect tissue as in Experiment 2.1 and analyze for FJ staining indicative of cell injury. The results from this experiment will be compared with those of Experiment 2.1 to determine whether 40% reduction of blood flow is sufficient to cause call injury.

Expected Results: Based on our preliminary results, we predict that a 40% reduction of CBF in the absence of TBI will not be sufficient to cause a significant amount of cell injury.

Possible pitfalls, alternative approaches: As previously discussed, ET-1 can elicit direct effects on neurons and, hence, ET-1 injections may cause changes in neuronal integrity independent of CBF changes. However, it should be pointed out that the consensus of literature suggests that ET-1 participates in both cell death and

Expected Results: We expect that, similarly to the previously shown ETrB mediation of ET-1 induced vasodilation, BQ-788 will not reduce the extent and duration of vasoconstriction after TBI and may even enhance TBI-mediated vasoconstriction.

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Possible pitfalls, alternative approaches: Same as in Experiment 1.5.

Experiment 1.7: Determine the effect of BQ-123, an ETrA antagonist, on CBF following TBI.
Animals (N=32) will be treated as in Experiment 1.4, however various doses of IV injection of BQ-123 (0.1mg/kg, 1.0mg/kg, 10mg/kg) will be administered (N=8 per group) 30min following TBI. 4 hours following TBI, animals will be re-anesthetized and another sequence of MRI CBF measurements will be taken. This will be repeated at 24 and 48 hours after injury. CBF will be analyzed in smCx and Hipp of all scans and will be compared to results in Experiment 1.4 using ANOVA with LSD posthoc to determine the effect of BQ-123 on CBF following TBI.

Expected Results: We expect, based on preliminary data, that BQ-123 will block TBI-mediated decreases in CBF.

Possible pitfalls, alternative approaches: Based on the provided preliminary data, we do not predict any problems with this experiment. However it may be challenging to interpret conflicting data from experiments 1.5 and 1.7. That is, how does one interpret the possibility that no observable vasoconstriction is detected when CBF is decreased? If this occurs, given the limits of the EM quantification of luminal area, we would rely on the MRI data as a more accurate predictor of enhanced vascular reactivity. To rule out the possibility that overall blood pressure is compromised and hence overall CBF diminished, Dr. Mueller, Assistant Professor in the Department of Physiology will assist us in taking mean arterial pressure (MAP) recordings of all animals. *Further, though our preliminary data seems to suggest that one injection is sufficient to ameliorate TBI-induced hypoperfusion, it may be necessary to incorporate a multiple-injection paradigm to maintain control level CBF. Therefore, we are prepared to give injections a 30 min, 24.5 hrs and 48.5 hrs post TBI in the following groups: BQ-123+veh+veh, BQ-123+BQ-123+veh, BQ-123+BQ-123+BQ-123. 30 min after each injection we will measure CBF to determine whether we can maintain control level CBF.*

Experiment 1.8: Determine the effect of BQ-788, an ETrB antagonist, on CBF following TBI.
Animals (N=32) will be treated as in Experiment 1.4, however various doses of IV injection of BQ-788 (0.01mg/kg, 0.1mg/kg, 1.0mg/kg) will be administered (N=8 per group) 30min following TBI. 4 hours following TBI, animals will be re-anesthetized and another sequence of MRI CBF measurements will be taken. This will be repeated at 24 and 48 hours after injury. CBF will be analyzed in smCx and Hipp of all scans and will be compared to results in Experiment 1.4 using ANOVA with LSD posthoc to determine the effect of BQ-788 on CBF following TBI.

Expected Results: We expect, based on preliminary data, that BQ-788 will not block TBI-mediated decreases in CBF.

Possible pitfalls, alternative approaches: In addition to the caveats addressed in Experiment 1.7, our preliminary data suggests a trend (most prevalent in Hipp) towards a further decrease in CBF following BQ-788 treatment which would be expected based on its ability to block vasodilation. Therefore, we could utilize higher doses to test the effects of exacerbation of hypoperfusion following TBI. However, while this would be interesting in theory, in our preliminary trials, doses higher than 1.0mg/kg led to severe adverse effects in our animals including neurologic deficits, which compromised the interpretation of all results.

SPECIFIC AIM 2 tests the hypothesis that decreased CBF following TBI causes secondary neuronal injury following TBI.

Rationale

Principal Investigator/Program Director (Last, First, Middle): Kreipke, Christian W.

Experiment 1.3: Determine the extent of vasoconstriction in smCx and Hipp at 4, 24 and 48h post injury.
Animals will be treated as in Experiment 1.1, however tissue will be processed for electron microscopy (EM). Using a technique already published by our laboratory (Rafols et al., 2007b), we will quantify luminal area of cross-sectioned microvessels from 1μm thick plastic and ultrathin sections for EM from the smCx and Hipp to determine the extent of vasoconstriction.

Expected Results: We expect that TBI will result in a significant increase in vasoconstriction (i.e., a significant decrease in luminal area) which temporally coincides with the observed increase in ET-1 expression following injury.

Possible pitfalls, alternative approaches: We recognize that determining luminal area from EM can be difficult and the risk of sampling error is possible. However, we do not anticipate any major difficulties, here, since Dr. Rafols has over 30 years of experience in observing and quantifying EM data.

Experiment 1.4: Measure CBF in smCx and Hipp at -4, 4, 24 and 48h post injury using ASL-MRI.
Animals will be placed into a 4.7T MRI and blood flow measurements will be taken. 4 hours after completion of MRI scans (to allow for the effects of anesthetic to subside) TBI will be induced as described in General Methods. 4 hours following TBI, animals will be re-anesthetized and another sequence of ALS-MRI CBF measurements will be taken. This will be repeated at 24 and 48 hours after injury. CBF will be analyzed in smCx and Hipp of all scans and will be compared across time points to determine the effect of TBI on CBF.

Expected Results: Based on preliminary results, we expect that TBI will significantly decrease CBF in both smCx and Hipp at all time points following injury.

Possible pitfalls, alternative approaches: We have previously used ketamine/xylazine as the anesthetic agent which is known to affect oxygen saturation and brain metabolism. Therefore, we have changed to continual infusion of halothane using a face mask modified for use in MRI. Since we have made this change, in conjunction with our previously published work and preliminary data shown here, we do not predict any significant problems with this experiment.

Experiment 1.5: Determine the effect of BQ-123, an ETrA antagonist, on vasoconstriction following TBI.
Animals will be treated as in Experiment 1.3, however various doses of IV injection of BQ-123 (0.1mg/kg, 1.0mg/kg, 10mg/kg) will be administered (N=6 per group) 30min following TBI. Luminal area will be determined from EM and results will be compared to those in Experiment 1.3 using ANOVA with LSD posthoc to determine the effect of BQ-123 on TBI-mediated vasoconstriction.

Expected Results: We expect that, as ETrA has been shown to mediate ET-1-elicited vasoconstriction, BQ-123 will reduce the extent and duration of vasoconstriction after TBI.

Possible pitfalls, alternative approaches: Again we acknowledge that determining luminal area of blood vessels using EM is challenging. However, again we defer to the expertise of Dr. Rafols who has over 30 years experience in working with EM.

Experiment 1.6: Determine the effect of BQ-788, an ETrB antagonist, on vasoconstriction following TBI.
Animals will be treated as in Experiment 1.3, however various doses of IV injection of BQ-788 (0.01mg/kg, 0.1mg/kg, 1.0mg/kg) will be administered (N=6 per group) 30min following TBI. Luminal area will be determined from EM and results will be compared to those in Experiment 1.3 using ANOVA with LSD posthoc to determine the effect of BQ-788 on TBI-mediated vasoconstriction.